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THE UNIVERSITY OF ALBERTA

POTASSIUM EVOKED RELEASE OF NORADRENALINE FROM RAT HEARTS

by

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A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHARMACOLOGY

EDMONTON, ALBERTA, CANADA
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THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled *POTASSIUM EVOKED RELEASE OF NORADRENALINE FROM RAT HEARTS* submitted by John Richard Carpenter in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Pharmacology.

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ABSTRACT

Isolated rat hearts were perfused with a Krebs/bicarbonate solution and labelled by infusing ^{3}H-NA at a concentration of 10^{-7} M. The efflux of radioactivity was determined by liquid scintillation spectrometry of effluent samples. After 60 min efflux, perfusion with a Krebs solution containing an additional 56 mmol KCl per litre caused a marked increase in efflux of radioactivity. Equally hyperosmolar sucrose and choline chloride did not alter the efflux pattern significantly, whereas 56 mM KCH_3SO_4 induced a release of radioactivity of the same magnitude as that evoked by 56 mM KCl indicating that the release was independent of the osmolarity or the Cl^- ion. Perfusion with varying concentrations of KCl revealed that release of radioactivity was related to $\log[K^+]$ at K^+ concentrations higher than a threshold level of about 35 mM. Perfusion with a solution containing 56 mM NaCl or $NaCH_3SO_4$ produced an increased release of radioactivity which did not differ from that induced by KCl, while 56 mM LiCl produced an intermediate release, indicating that release may be initiated by monovalent cations by mechanisms other than depolarization of sympathetic nerves. Chemical sympathectomy with 6-OHDA and "pharmacological" sympathectomy with reserpine reduced the ability of hearts to retain radioactivity (measured after 60 min perfusion) to 9% and 3% of normal respectively, but these treatments only reduced the KCl induced release to 50% and 45% of normal respectively, indicating that an intact, functional sympathetic nervous system was only responsible for between 50% and 60% of the KCl induced release. Treatment with the $Uptake_2$ inhibitor SKF-550 and the $Uptake_1$ inhibitor DMI, following pretreatment with 6-OHDA did not significantly alter the KCl induced release, indicating that uptake into the site from which KCl causes release was

not by means of the Uptake$_2$ process and confirming that after 6-OHDA release from nerves was insignificant. When normal hearts were perfused with a solution containing SKF-550, the release induced by 56 mM KCl was not significantly different from that evoked from untreated hearts, confirming that the release site was independent of Uptake$_2$.

Elevated levels of K^+ were found to partially inhibit uptake of ^3H-NA, indicating that increased efflux of radioactivity seen when hearts were perfused with 56 mM KCl was due in part to an inhibition of the re-uptake of both spontaneously released ^3H-NA and that ^3H-NA released by KCl *per se*.

It is proposed that in addition to being able to stimulate release of sympathetic transmitter by depolarizing sympathetic nerves, K^+ is able to induce release of either NA or cationic metabolites of NA from an extraneuronal, possible extracellular, site by means of an ion-exchange process. Consequently, it is further proposed that the results of experiments in which transmitter release from sympathetically innervated tissues is evoked by KCl should be interpreted with caution, bearing in mind that only 50-60% of the released transmitter is of neuronal origin.

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I wish also to thank my supervisor, Dr. C.W. Nash and also Dr. J. S. Charnock, Dr. D.A. Cook and Dr. D. M. Paton for their invaluable advice and encouragement.

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INTRODUCTION

Many pharmacological studies of neurotransmitter mechanisms have involved the use of elevated potassium to induce transmitter release and in almost all of these studies, it is assumed that elevated levels of potassium produce specific effects at nerve membranes which result in transmitter release in a manner very similar to normal, physiological release (Liley, 1956; Douglas and Rubin, 1961; Haeusler, Thoenen, Haefely and Hurliman, 1968; Hubbard and Kwanbunbumpen, 1968; Kirpekar and Wakade, 1968; Bisby and Fillenz, 1969; Harris and Roth, 1971; Hopkin and Neal, 1971; Vogel, Silberstein, Berv and Kopin, 1972; Nash, Taylor and Drouin, 1972; Sorimachi, Oesch and Thoenen, 1973; Stjärne, 1973; Zanella and Rall, 1973).

In a preliminary study (Carpenter and Nash, 1971) release of tritiated noradrenaline (^{3}H-NA) from perfused rat hearts was induced by 56 mM KCl, the assumption being that by raising the K^+ level in the perfusion fluid, the sympathetic nerves in the heart were depolarized and that this depolarization triggered the release of ^{3}H-NA from transmitter stores in the sympathetic nerves in a more or less physiological manner (Nash *et al.*, 1972). The object of the study presented here was to investigate the manner in which KCl causes release of ^{3}H-NA from the labelled sympathetic transmitter stores of perfused rat hearts and to determine whether it is valid to use this manouever to simulate electrically stimulated release of transmitter.

In order to put this study in perspective, it will be prefaced by a brief account of current concepts regarding the structure, functioning and pharmacology of adrenergic nerves.

MORPHOLOGY

The typical peripheral adrenergic neurone in the sympathetic nervous system has a moderate sized cell body, [on average less than 25 µm (Bloom, 1972)] located in the paravertebral chain of ganglia and a long, unmyelinated axon, generally a C-fibre (Haefely, 1972). Unlike the motorneurone, the adrenergic neurone does not have (a) terminal bouton(s) making close contact with a specialized area of the end organ it innervates. Rather, terminal adrenergic neurones break up into a plexus of fine fibres, spreading over the surface of the end organ. These terminal fibres are of the order of 0.1-0.2 µm in diameter and a particularly conspicuous feature of this plexus is the presence of "beads" or "varicosities" about 1-2 µm in diameter every 3-10 µm along the terminal fibres (Garven and Gairns, 1952; Hillarp, 1959; Norberg and Hamberger, 1964; Malmfors, 1965; Merilees, 1968; Bloom, 1972). These varicosities make the closest approach to the innervated cells and although these latter show no specialization of membrane, it is highly likely that the varicosities represent the sites of transmitter release. These series/parallel nerve fibre networks have been described as making "en passage" contacts with the effector cells (Grillo, 1966; Iversen, 1967).

Electron microscopy reveals that the varicosities are packed with small, dense-cored, vesicular structures, whilst in the inter-varicose regions there are relatively few vesicles (Burnstock, 1970). When the diameters of the vesicles are measured and tabulated, a bimodal frequency distribution is revealed, one hump comprising vesicles of 40-60 nm and the second hump vesicles of 80-120 nm in diameter (Grillo and Pallay, 1962). These vesicles are referred to as small and large (dense-cored) vesicles, the large vesicles constituting about 1-5% of the total (Bondareff, 1965; van Orden, Bloom, Barrnett and Giarman, 1966; van Orden, Bensch and Giarman, 1967a; van Orden, Bensch, Langer and Trendelenburg, 1967b; Farrel, 1968; Geffen and Ostberg, 1969).

That the dense-core vesicles seen with the electron microscope represent the storage sites for the sympathetic transmitter (NA) was first suggested by de Robertis and Pellegrino de Iraldi (1961) and this hypothesis is supported by several findings:-

(a) dense-cored vesicles are common in electron micrographs showing the nerves of the dilator muscle of the rat iris, a predominantly sympathetically innervated organ, whereas the constrictor muscle, which has a predominantly parasympathetic innervation, shows only clear-cored vesicles (Richardson, 1964),

(b) when tissues or organs are sympathetically denervated, the decline in NA content parallels the disappearance of dense-cored vesicles (van Orden *et al.*, 1967a),

(c) pharmacological depletion of tissues of NA results in the disappearance of the electron opaque cores of the vesicles, whilst subsequent restoration of functional tissue NA is accompanied by a return of the dense cores to the vesicles (van Orden *et al.*, 1966; van Orden *et al.*, 1967a).

Further support is provided by studies on the embryologically related adrenal medulla. In 1953, Blaschko and Welch and also Hillarp, Lagerstedt and Nilsson prepared fractions from homogenates of adrenal medullae in which the bulk of the catecholamine content was associated with a granular fraction and it is now quite clearly established that these "chromaffin granules" are the storage sites for catecholamines in the adrenal medulla (Kirshner, 1969; Smith, 1968). Von Euler and

Hillarp (1956) were able to prepare a fraction from bovine splenic nerves which contained particle bound NA and similar NA containing fractions have since been prepared from a variety of sympathetically innervated organs, so that it is now well accepted that the bulk of NA in sympathetic neurones is stored in vesicles. (For an extensive bibliography see Geffen and Livett, 1971; Smith, 1972). It is not yet clear whether significant amounts of NA exist in a free cytoplasmic pool in the normal, intact, sympathetic neurone or whether all the transmitter is located in vesicles. In preparations of isolated vesicles, seldom more than 75% of the NA is found bound to the vesicles, although von Euler (1967) was able to obtain vesicle preparations in which 80-90% of the NA was vesicle bound. Whilst it is likely that the vigorous disruptive techniques necessary to break down the nerves are also sufficient to cause damage to a significant fraction of vesicles, the existence of a free transmitter pool is still a matter of some debate.

STORAGE VESICLES

It has been estimated that the concentration of catecholamines in adrenal medullary vesicles (chromaffin granules) is of the order of 0.5 M (Hillarp and Nilsson, 1954) and while there are no reports which suggest that this value pertains to sympathetic nerve vesicles it would seem unlikely that a value many orders of magnitude less pertains. How, then, can these vesicles maintain such a high concentration of NA? A convenient approach to this problem is to examine the properties of

(a) the vesicle membrane

(b) the vesicle contents.

(a) *Properties of the Vesicle Membrane*

In high power electron micrographs of noradrenergic nerves stained with osmium tetroxide, the vesicles appear to be bound by a bi-layer, not dissimilar in appearance from plasma membrane, and to have a separate core, the total structure resembling that of chromaffin granules from adrenal medulla (Sjöstrand and Wetzstein, 1956; de Robertis and vaz Ferreira, 1957 a,b; Banks, 1965; de Robertis, Pellegrino de Iraldi, Rodríguez de Lores Arnaiz and Zieher, 1965; Fillenz, 1971; Bloom, 1972).

Isolated chromaffin vesicles, in contrast to sympathetic nerve vesicles, can be prepared to a very high degree of purity and biochemical analysis shows a very large proportion of lipid materials, suggesting that these vesicles have a lipid containing membrane (Smith, 1968). Unfortunately the contamination of isolated nerve vesicle preparations by other membranous material, particularly mitochondria and microsomes, militates against ascribing their high lipid content to an origin in vesicular membranes. It is conceivable that in preparation of the highest purity, a difference, either quantitative or qualitative in the lipid contents of vesicle preparations made from normal tissues and sympathectomized tissues might be revealed, but to date, no such study appears to have been performed.

Isolated vesicle preparations from adrenal medullae were shown to accumulate catecholamines by a process that required adenosine triphosphate (ATP) and Mg^{++} ions (Kirshner, 1962; Carlsson, Hillarp and Waldeck, 1963; Stjärne, 1964). Similarly, preparations of vesicles from sympathetic nerves have been shown to accumulate NA by an ATP, Mg^{++} dependent process (von Euler, Stjärne and Lishajko, 1963; von Euler and Lishajko, 1964; Stjärne, 1964; Stjärne and von Euler, 1965; Lishajko,

1969) which is suggestive of an energy requiring process. This is supported by the work of von Euler and Lishajko (1969) who showed that a number of metabolic inhibitors blocked this uptake process, whilst von Euler (1970) extended this work and suggested that the uptake process required an ATPase associated with the vesicle membrane. One way, then, that the vesicles could maintain their high content of NA would be by means of an inwardly directed transport mechanism.

(b) Properties of the Vesicle Contents

When isolated vesicles are allowed to accumulate radioactively labelled NA and are then incubated in the absence of ATP and Mg^{++}, they rapidly lose NA, which, when considered with the evidence given above, suggests a simple pump/leak arrangement for NA storage. However, there is evidence of an intravesicular sequestration mechanism, so that in fact the bulk of intravesicular noradrenaline may be bound so that there is a down-hill concentration gradient into the vesicles. This situation probably pertains in the case of adrenal medullary vesicles, analyses of which have revealed strikingly high contents of adenine nucleotides, especially ATP, amounting to as much of 15% of the dry weight of such isolated vesicle preparations (Smith, 1968). When the relative molar contents of catecholamines and nucleotides are compared, a ratio close to 4:1 is found in most of the species studied, which suggests that catecholamines may be stored in the vesicles in the form of a complex with ATP. Supportive evidence for this was provided by Berneis, Pletscher and da Prada (1969, 1970), who showed that the addition of critical amounts of divalent alkaline earth metals, especially Ca^{++} and Mg^{++} to solutions of NA and ATP (3:1 molar ratio), led to the formation of aggregates with increased apparent molecular weights. Further evidence for the existence of a catecholamine-ATP storage complex is that stimulation of the adrenal medulla leads to the release of catecholamines and ATP metabolites in approximately the same molar ratio as they are found in isolated medullary vesicle preparations (Douglas, Poisner and Rubin, 1965; Douglas and Poisner, 1966; Banks, 1966).

As discussed above, biochemical analyses of nerve vesicle preparations are hampered by the lack of purity of these preparations. However, the ratio of catecholamines to ATP also appears to be close to 4:1, although there is considerable variation between the values found in the different studies (Schümann, 1958; von Euler, Lishajko and Stjärne, 1963; Potter and Axelrod, 1963c; de Potter, Smith and Schaepdryver, 1970; Lagercrantz, 1971).

Another feature of adrenal medullary vesicles is their high content of water soluble proteins (Hillarp and Nilsson, 1954; Blaschko, Born, D'Iorio and Eade, 1956). These proteins are of two major types, (a) proteins with no enzymic properties, (b) proteins with enzymic properties.

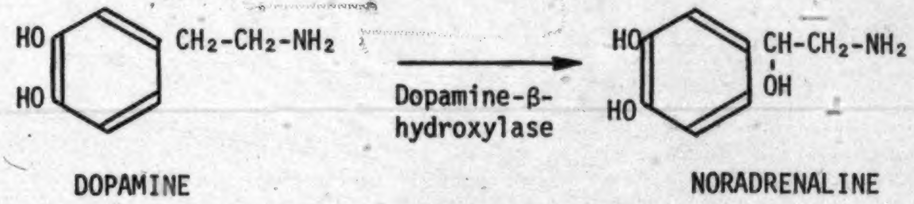
Non-Enzymic Soluble Proteins. The soluble proteins in medullary chromaffin granules or vesicles have been termed the chromogranins and analysis reveals some eight components, with one component predominating (40% of the total). This form of the protein has been called chromogranin A (Schneider, Smith and Winkler, 1967).

Chromogranin A is a highly acidic protein with a molecular weight of about 75,000, composed of two submits each with a molecular weight of about 40,000 (Smith and Kirshner, 1967; Smith and Winkler, 1967). Hillarp (1958) suggested that the presence of chromogranins in storage vesicles rendered the catecholamine/ATP complex less diffusible, but it was found that chromogranin A cannot bind significant amounts of catecholamines, either with or without ATP and Mg^{++} (Smith and Kirshner, 1967). Furthermore, Smith (1968) has calculated that, in the adrenal medulla at least, the large decrease in entropy which would result from the formation of a chemical complex between chromogranin A, ATP and adrenaline in the proportions in which they are found in the vesicles, could only occur if compensated for by an equivalent decrease in enthalpy of the same order as would result from the formation of covalent bonds. Such a situation would clearly make the process of release of free adrenaline complex indeed. Smith (1968) goes on to suggest, however, that as circumstantial evidence suggests that chromogranin A exists in the form of a random coil (Smith and Winkler, 1967), the ATP-catecholamine complex could be rendered less diffusible by being trapped within the coils of the protein when this latter is in a gel form. The diffusibility of the complex could conceivably be altered by the swelling or contracting of the gel according to its ionic environment. It is also possible that the highly ionic environment within the interstices of the chromogranin molecule favours the formation of the micellar complex of ATP and catecholamines proposed by Berneis *et al.* (1969, 1970).

In 1965, Banks and Helle were able to produce an antiserum to purified chromogranin obtained from isolated medullary vesicles and were able to use this to show that chromogranins were released from the adrenal medulla on stimulation, along with catecholamines and ATP. This led to the exciting findings that antiserum to adrenal medullary chromogranins reacted with sympathetic nerves (Hopwood, 1968) and isolated splenic nerve vesicles (de Potter, Schaepdryver, Moerman and

Smith, 1969b). It has also been shown that chromogranins are present in sympathetically innervated tissues in the same distribution as NA by comparing micrographs of such tissues stained with either an immunofluorescent dye specific for chromogranins or by the formaldehyde condensation fluorescence method of Falck (1962) which demonstrates NA (Livett, Geffen and Rush, 1968; Geffen, Livett and Rush, 1969a, b; Livett, 1970).

Enzymic Protein. The second major soluble protein component of isolated medullary vesicles is the enzyme dopamine-β-hydroxylase (Potter and Axelrod, 1963b; Potter, 1967; Austin, Livett and Chubb, 1967; Stjärne and Lishajko, 1967; Oka, Kajikawa, Ohuchi, Yoshida and Imaizumi, 1967; Laduron and Belpaire, 1968; Viveros, Arqueros and Kirshner, 1968; Hörtnagl, Hörtnagl and Winkler, 1969). This enzyme catalyses the β-oxidation of Dopamine to NA:-



By showing that this reaction could be prevented in the whole tissue by reserpine but not in lysed vesicle preparations it was demonstrated that the enzyme was intravesicular, as reserpine blocks the uptake of NA and dopamine into vesicles (Stjärne and Lishajko, 1966; Rutledge and Weiner, 1967).

The soluble proteins of storage vesicles, then, can contribute to the maintenance of a high intravesicular concentration in two ways, firstly by decreasing the diffusibility of the catecholamine or catecholamine/ATP complex and secondly by the de novo synthesis of NA within the vesicle.

FUNCTIONING OF THE NORADRENERGIC NERVOUS SYSTEM

When a substance is proposed as a physiological neurotransmitter, three important criteria must be met before the chemical can be accepted as a genuine transmitter. An examination of these criteria as applied to NA provides a convenient summary of our present concepts of noradrenergic nerve function. These criteria are:-

(a) It must be possible to demonstrate an association between noradrenaline and sympathetic nerves.

(b) A synthetic system for NA in sympathetically innervated tissues must be shown.

(c) There must be an adequate mechanism for terminating the action of released NA.

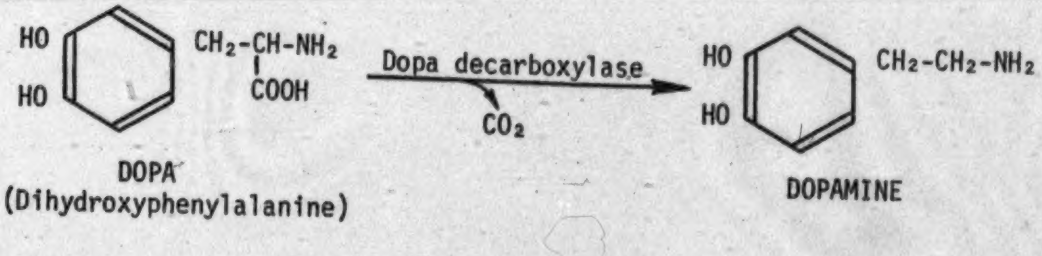
(a) *Noradrenaline in Sympathetic Nerves*

Rexed and von Euler (1951) showed that the NA content of nerve trunks is closely related to the proportion of non-myelinated, autonomic fibres in the trunk. The splenic nerve, which is almost entirely composed of non-myelinated C-fibres, has the highest NA content of any nerve studied, which correlates well with the known, rich, sympathetic innervation of the spleen (von Euler, 1956). In fact, it can be shown that the NA content of many organs and glands is related to their sympathetic innervation and that denervation, or more specifically sympathetic denervation, leads to a disappearance of both NA and function (von Euler and Purkhold, 1951; Goodall, 1951).

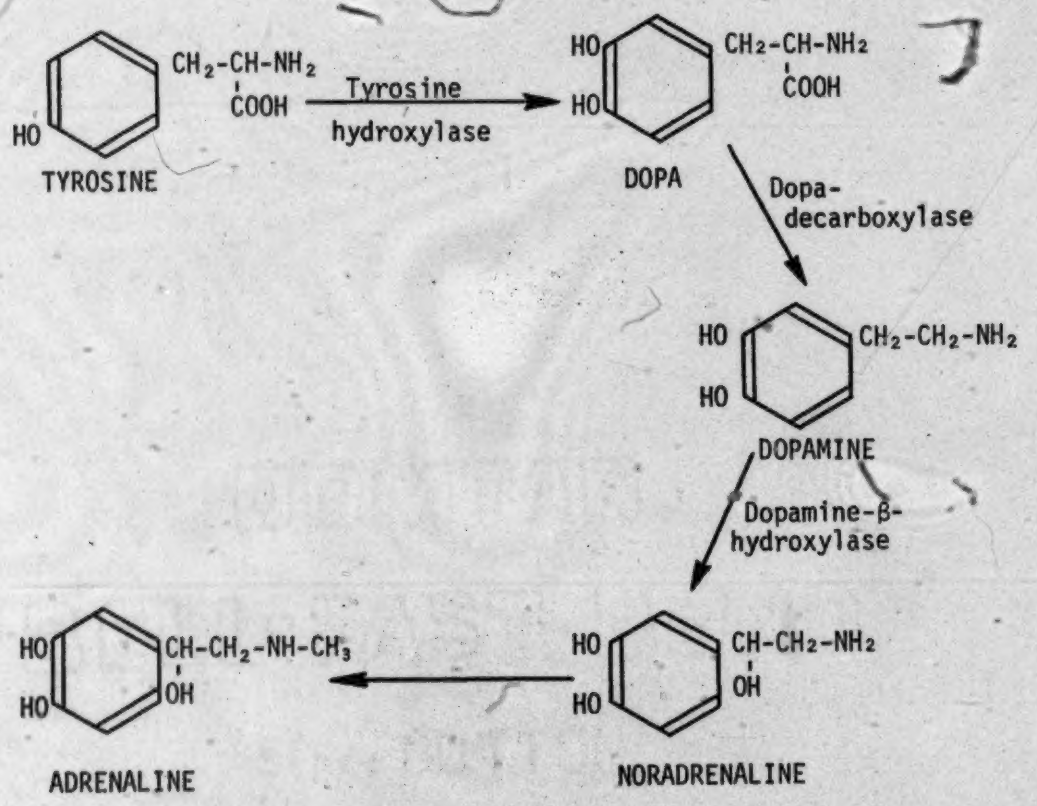
With the development of the formaldehyde condensation fluorescence histochemical method of Falck for the demonstration of mono-amines, (Falck, 1962; Dahlström and Fuxe, 1964; Falck and Owman, 1965) it became clear that there was a dense network of fine, noradrenaline containing fibres in sympathetically innervated tissues, the fluorescence pattern closely resembling the description of sympathetic nerve fibres made by Hillarp using silver staining techniques (Hillarp, 1959). This fluorescence was found to disappear on denervation, immunosympathectomy and with drug treatments that depleted tissues of NA.

(b) *Synthesis of Noradrenaline*

In 1938, Holtz, Heise and Lüdtke reported the existence of an enzyme capable of converting L-DOPA to dopamine, to which the name dopa decarboxylase was given.



Shortly after this discovery, Blaschko (1938) proposed a theoretical series of reactions by which adrenaline and noradrenaline could be synthesized from the amino acid tyrosine.


TYROSINE
Tyrosine hydroxylase
DOPA
Dopa-decarboxylase
DOPAMINE
Dopamine-β-hydroxylase
NORADRENALINE
ADRENALINE
HO
CH₂-CH-NH₂
COOH
CH₂-CH₂-NH₂
CH-CH₂-NH₂
OH
CH-CH₂-NH-CH₃

This remarkably prophetic scheme has proven to be essentially accurate, the full scheme being first confirmed using radioactively labelled tyrosine and adrenal medulla (Demis, Blaschko and Welch, 1955; Hagen, 1956; Kirshner and Goodall, 1956; Masuoka, Schott, Akawie and Clark, 1956; Udenfriend and Wyngaarden, 1956; Pellerin and D'Iorio, 1957). The demonstration of this pathway in sympathetic nerves is made technically more difficult by the relatively low "concentration" of sympathetic nerves in sympathetically innervated tissues, but in 1958, Goodall and Kirshner were able to convert labelled tyrosine to NA using homogenates prepared from sympathetic nerves and ganglia, a similar demonstration being made later using brain slices by Masuoka, Clark and Schott (1961) and Masuoka, Schott and Petriello (1963). This conversion has since been shown in many sympathetically innervated tissues and organs as the availability of labelled tyrosine of high specific activity has increased. It has also been found that this synthetic pathway is seriously impaired following sympathetic denervation, showing that at least one step in the pathway requires the presence of sympathetic nerves (Carlsson and Waldeck, 1963; Musacchio and Goldstein, 1963; Spector, Sjoerdsma, Zaltman-Nirenberg, Levitt and Udenfriend, 1963; Fischer, Musacchio, Kopin and Axelrod, 1964; Anden, Magnusson and Rosengren, 1965; Klingman, 1965; Potter, Cooper, Willman and Wolfe, 1965; Iversen, Glowinski and Axelrod, 1966).

The first enzyme in the pathway, tyrosine hydroxylase, was shown to be present in axoplasm (Nagatsu, Levitt and Udenfriend, 1964) and to require tetrahydropyridines and divalent iron for activity (Ikeda, Levitt and Udenfriend, 1965). This enzyme is not totally specific, as it will also catalyze the conversion of phenylalanine to tyrosine, so that either tyrosine or phenylalanine may be the starting point of NA synthesis. An important property of ~~tyrosine~~ hydroxylase is that it is inhibited by DOPA and by NA and as it is the rate limiting step in the pathway, this represents a possible feedback control mechanism for the regulation of synthetic rate (Spector *et al.*, 1963; Levitt, Spector, Sjoerdsma and Udenfriend, 1965; Nagatsu *et al.*, 1964).

The second enzyme, DOPA-decarboxylase has been found in all tissues which contain adrenergic neurones or chromaffin cells (Holtz, Credner and Koepp, 1942; Langeman, 1951) and is present in the axoplasm of adrenergic nerves (Stjärne, 1966). It requires pyridoxal-5'-phosphate as a co-factor (Holtz and Palm, 1964).

The third enzyme, dopamine-β-hydroxylase, which was discussed briefly above, was first prepared from adrenal medulla and has been shown to contain copper and to require ascorbate and fumarate as co-factors (Levin, Levenberg and Kaufman, 1960; Goldstein, Lauber and McKereghan, 1965; Kaufman and Friedman, 1965). Unlike the two preceding enzymes, which are present in the axoplasm of adrenergic nerves, dopamine-β-hydroxylase is confined to storage vesicles (von Euler and Lishajko, 1968; Stjärne, Roth and Lishajko, 1967 ; Potter and Axelrod, 1963a,b,c; Stjärne and Lishajko, 1967).

It is clear, then, that sympathetic adrenergic nerves possess an adequate synthetic mechanism for NA and in addition, a mechanism which is capable of regulation via a simple secondary product feedback inhibition.

(c) *Termination of Action*

In 1928, Hare described an enzymic oxidation of tyramine by liver and similar reports of an "amine Oxidase" were made by Blaschko, Richter and Schlossman (1937) and Richter (1937). With better characterization this enzyme became known as monoamine oxidase (MAO) to distinguish it from enzymes which catalyse the oxidation of diamines, such as histamine.

This enzyme, MAO, has been shown to be very widely distributed throughout many tissues, not to be especially associated with sympathetically innervated tissues, to be apparently associated with mitochondrial membranes and not to be especially specific for catecholamines (Blaschko *et al.*, 1937; Blaschko, 1952; Hawkins, 1952; Pratesi and Blaschko, 1959; de Lores Arnaiz and de Robertis, 1962).

In cholinergic systems, the action of released acetylcholine is terminated by its rapid hydrolysis by the enzyme acetylcholinesterase and by analogy with the cholinergic system, it is tempting to confer this important function upon MAO in adrenergic systems. This view, however, was complicated by the discovery of a second enzyme which could degrade catecholamines. This was catechol-O-methyl transferase (COMT) (Axelrod, 1957). Like MAO, COMT has proven to be widely distributed and not to be located only in or near adrenergic fibres. When potent inhibitors of MAO and COMT became available, the relative contributions of these two enzymes toward the termination of action of both exogenous and endogenous NA could be investigated. Brown and Gillespie (1957) could show no increase in the outflow of NA from perfused, stimulated cat spleen when MAO was inhibited. Crout (1961) was unable to show any potentiation or prolongation of the action of NA when both COMT and MAO were inhibited. Consequently, some other mechanism for dealing with released NA had to be found and this turned out to be re-uptake of the transmitter by adrenergic nerves.

With the advent of radioactively labelled catecholamines of high specific activity, it became apparent that many tissues could accumulate catecholamines (Axelrod, Weil-Malherbe and Tomchick, 1959; Whitby, Axelrod, and Weil-Malherbe, 1961; see also Iversen, 1967). The evidence in favour of the hypothesis that uptake occurs into sympathetic nerves can be summarized as follows:-

(i) Correlation between uptake and sympathetic innervation. The first evidence of this kind was that the amount of uptake of radioactive NA by various tissues was related to the endogenous NA content of the tissue, which is an indication of the degree of sympath-

etic innervation (Whitby *et al.*, 1961; Crout, 1964). More sophisticated studies, taking differences in blood flows into account, have shown much closer correlations than these original reports (Wurtman, Kopin, Horst and Fischer, 1964; Kopin, Gordon and Horst, 1965).

(ii) Dependence on intact sympathetic innervation. The uptake of labelled catecholamines has been shown to be grossly impaired following procedures which reduce the integrity of the sympathetic nervous system, *e.g.*, surgical, immunological and chemical sympathectomy (Hertting, Axelrod, Kopin and Whitby, 1961a; Potter *et al.*, 1965; Iversen, 1965; Zaimis, Berk and Callingham, 1965; Thoenen and Tranzer, 1968). In addition, the reappearance of a functional sympathetic innervation is accompanied by a return of endogenous tissue NA levels and the reappearance of catecholamine uptake.

(iii) Autoradiography. The ability of autoradiographic techniques to localize labelled materials is limited by the diffusion characteristics of the material, the grain size of the emulsion and the path length of the emitted particle. However, electronmicroscopic autoradiography of tissues which had been exposed to ^{3}H-NA has shown a clear association between exposed silver grains and postganglionic sympathetic nerve terminals (Wolfe, Potter, Richardson and Axelrod, 1962; Wolfe and Potter, 1963). As the resolution of autoradiographs of ^{3}H is hardly any better than the dimensions of adrenergic nerve terminals, little more information on uptake sites can be expected to come from such studies.

(iv) Fluorescence microscopy. Using the formaldehyde condensation fluorescence method suggested by Falck, 1962; Dahlström

and Fuxe, 1964; Falck and Owman, 1965, it has been possible to show that the fluorescent appearance (*i.e.*, NA content) of depleted fibres could be restored by exposure to large concentrations of NA (Hamberger, Malmfors, Norberg and Sachs, 1964). In addition, Gillespie and Kirpekar (1965a,b) combined fluorescence microscopy with autoradiography to show that ^3H could be associated with NA containing fibres following exposure of tissues to ^3H-NA.

(v) Correlation with functional stores. When a tissue that had accumulated ^3H-NA was stimulated, it was found that the specific activity of the released NA was lower than that of the originally infused ^3H-NA (Hertting and Axelrod, 1961; Gillespie and Kirpekar, 1965a,b). This implies that the ^3H-NA had been taken up into a storage site from which release could occur and in which it was able to undergo some degree of mixing with the endogenous store of unlabelled NA. When the rate of loss of ^3H-NA from tissues or organs was followed, Hertting and Axelrod (1961) found that procedures which diminished activity in the postganglionic sympathetic nerves, *e.g.*, decentralization or treatment with ganglion blocking drugs, resulted in decreases in the rate at which ^3H-NA disappeared from the tissues. This further supports the hypothesis that the sites into which uptake of exogenous NA occurs are also sites from which normal, physiological release of endogenous transmitter occurs. Subcellular fractionation has revealed that these sites are largely, if not totally represented by the storage vesicles discussed earlier (Potter and Axelrod, 1963a,b,c).

The current concensus is that re-uptake by noradrenergic nerves is largely responsible for the termination of action of endogenously released NA. This explains the findings that when the postganglionic nerves to perfused, sympathetically innervated organs are stimulated at physiological frequencies (<10 Hz; Folkow, 1952), little or no sympathetic transmitter can be detected in the effluent, whilst stimulation at grossly unphysiological rates leads to marked outflow of transmitter (Celander, 1954; Brown and Gillespie, 1957; Stinson, 1961; Thoenen, Hurlimann and Haefely, 1964; Brown, 1965). In 1959, Koelle reviewed the possible mechanisms for the termination of action of released NA and came to the conclusion that "tissue redistribution" was the major factor. Brown (1965) has shown that more than 90% of the noradrenaline released from splenic nerve terminals is removed by tissue uptake, whilst more recently Geffen, Livett and Rush (1970a) have estimated that upwards of 70% of endogenously released NA is taken up and re-used by noradrenergic nerves. In addition to providing a means of terminating the action of released NA, the uptake process also enables the noradrenergic nerves to re-use much of their transmitter, the economic advantages of which are clear.

PROPERTIES OF THE UPTAKE PROCESS

As a result of detailed kinetic studies, Iversen was able to distinguish two uptake processes, with quite different kinetic properties and to which he gave the names Uptake$_1$ and Uptake$_2$ (Iversen, 1963; 1965; 1967). Uptake$_1$ is the process which is seen at low concentrations of catecholamines and is clearly a neuronal process. Uptake$_2$, on the other hand, only becomes apparent at higher concentrations of NA and since Iversen first reported the existence of this second uptake process (Iversen, 1965) a number of reports of uptake processes distinct from Uptake$_1$ have come from other laboratories, possibly all describing the same process, but possibly describing different processes (Hamberger, Norberg and Olson, 1967; Axelrod and Krokoff, 1967a; Eisenfeld, Landsberg and Axelrod, 1967b; Avakian and Gillespie, 1968; Simmonds and Gillis, 1968; Lightman and Iversen, 1969; Draskoczy and Trendelenburg, 1970; Gillespie, Hamilton and Hosie, 1970). Perhaps the only unanimous agreement between these workers would be that Iversen's original postulate, (Iversen, 1965) that Uptake$_2$ occurs into adrenergic nerves, is untenable. If this is so, it is then reasonable to discuss NA uptake under the headings "Neuronal Uptake" and "Non-neuronal Uptake" as, although the classical Uptake$_2$ as described by Iversen could well be occurring into adrenergic nerves, it seems clear that a similar process occurs into a variety of cellular types.

(a) *Neuronal Uptake*

The classical studies of Iversen (Iversen, 1963; 1965; 1966; 1967; 1971) have shown the neuronal uptake process to be saturable and to obey Michaelis-Menton kinetics. The affinity for NA is high and, in rat tissues at least, there is a marked stereoselectivity for the ℓ-isomer, (Iversen, 1967; Iversen, Jarrot and Simmonds, 1971; Hendley and Snyder, 1972) whilst the process is highly temperature sensitive and is inhibited by anoxia when combined with inhibitors of glycolysis (Hamberger, 1967; Paton, 1968; 1972; Wakade and Furchgott, 1968). These facts suggest a carrier mediated active transport system and Bogdanski and Brodie (1969) found the process to require extracellular sodium ions. As high extracellular concentrations of potassium ions inhibited uptake, they proposed a scheme for a carrier mediated uptake process derived from a model of Crane (1965) for sugar transport across intestinal epithelium. In essence, their model envisages NA binding to a membrane carrier to which Na^+ also binds (this latter process actually increasing the affinity for NA binding). This carrier-NA-Na^+ complex then diffuses down the concentration gradient into the nerve cell, where the high K^+ causes the NA, Na^+ and carrier to dissociate. The empty carrier then moves back to the outer surface of the membrane while the Na^+ is extruded from the cell by the membrane Na^+/K^+ pump. In other words the actual transport of NA is a passive process down a Na^+ concentration gradient. However, the inhibition of NA uptake by cardiac glycosides was found to be unrelated to their ability to inhibit the Na^+/K^+ pump, whilst manipulations of the ionic gradients failed to alter the uptake of NA in the manner predicted by the model. In addition, the affinity of the uptake mechanism for NA was not found to be altered by Na^+ or K^+, as would again have been predicted from the model (White and Keen, 1970; 1971; Keen and White, 1970; Paton, 1970; 1971; White and Paton, 1972). An alternative model has been proposed by White and Paton (1972) in which the carrier exists in two states in equilibrium with each other, one being inactive (X) and one active (X'). NA has the same affinity for the two forms but only X' is able to translocate across the nerve membrane. Na^+, however, has a higher affinity for X' than for X, whilst the resulting Na^+ - X' complex has the same affinity for NA as X or X' and the Na^+ - X' - NA complex the same translocation rate as X' - NA. The action of Na^+ in accelerating transport is brought about by the formation of the Na^+ - X' complex which shifts the equilibrium between X and X' in favour of X' so that more sites (X' and X' - Na^+) are available for NA transport. These authors have not, as yet, proposed a mechanism for the effects of K^+ on these transport mechanisms.

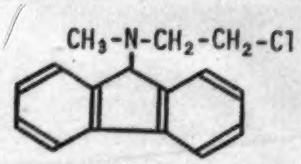
The structural requirements for neuronal uptake are quite strict and are, in essence, the absence of bulky N-substituents, the presence of at least one ring hydroxyl group (para- or meta-) and the absence of ring methoxy groups (Burgen and Iversen, 1965; Iversen, 1967).

Noradrenaline analogues which are taken up by this process also act as competitive inhibitors of noradrenaline uptake, their relative potencies as inhibitors being related to their affinities for the uptake carrier (Burgen and Iversen, 1965). However, the most potent inhibitors of neuronal uptake of NA have been found not to be noradrenaline analogues, but cocaine and desmethylimipramine (desipramine, DMI) (Iversen, 1965). Iversen reports that at a concentration of 10^{-6} M, DMI produced a 92.5% inhibition of NA uptake, whilst cocaine inhibited uptake by 95% at 10^{-5} M. Both of these agents have local anaesthetic properties and consequently one must be careful to ensure that the concentrations used for inhibiting NA uptake are not anaesthetic concentrations likely to interfere with the other functions of the adrenergic nerve terminals. The nerve blocking concentration for cocaine has been reported as 2.6×10^{-3} M (Skou, 1954) and for DMI as 6×10^{-3} M (Greef and Wagner, 1969) so that the concentrations of these agents which cause more than 90% inhibition of neuronal uptake are orders of magnitude lower than the concentrations which block nerve conduction.

(b) Non-Neuronal Uptake

In 1965, Iversen published evidence for the existence of a second catecholamine uptake process, for which he suggested the term

Uptake$_2$. He originally believed that this process only operated at high substrate concentrations, but more recent work has shown the process to occur at all substrate concentrations, its presence at low concentrations being masked by Uptake$_1$ and metabolic degradation of the substrate (Lightman and Iversen, 1969; Burnstock, McLean and Wright, 1971). Since the original description of Uptake$_2$, a number of workers have demonstrated accumulation of catecholamines in a variety of non-neuronal tissues (Hamberger *et al.*, 1967; Avakian and Gillespie, 1968; Simmonds and Gillis, 1968; Eisenfeld *et al.*, 1967a,b; Lightman and Iversen, 1969; Draskoczy and Trendelenburg, 1970; Gillespie *et al.*, 1970) and although the membrane processes which govern these accumulations may differ from cell type to cell type, they all clearly differ from Uptake$_1$. In general, the structural requirements for extraneuronal uptake are less rigorous than for neuronal uptake although clear rules appear to apply. Thus, in direct contrast to neuronal uptake, N-substitution and ring O-methylation favour extraneuronal uptake (Burgen and Iversen, 1965), so that normetanephrine acts as a powerful competitive antagonist of extraneuronal uptake (Burgen and Iversen, 1965). In studies of potential inhibitors of extraneuronal uptake, Iversen and his colleagues have shown that some β-haloalkylamines and some steroids are particularly good inhibitors (Iversen, Salt and Wilson, 1972; Iversen and Salt, 1970; Salt, 1972). They found that the most potent and selective inhibitor of extraneuronal uptake among the haloalkylamines they tested was the compound SKF-550 [N-(9-fluorenyl)-N-methyl-β-chlorethylamine)]:



Iversen uses the term IC50 to express the inhibitory potency of compounds and defines the term as the "drug concentration required to produce 50% inhibition of catecholamine uptake". The IC50 values for some of the more potent haloalkylamines and steroids are listed in Table 1.

It will be seen from Table 1 that SKF-550 is by far the most potent inhibitor of extraneuronal uptake of the four compounds listed and, with the possible exception of corticosterone, it has the largest selectivity ratio (500; selectivity ratio = IC50 Neuronal ÷ IC50 Extraneuronal) and it is therefore the obvious choice as an inhibitor of extraneuronal uptake. At a concentration of 10^{-6} M SKF-550, it would be expected that extraneuronal uptake would be inhibited by considerably more than 95%, whereas neuronal uptake would be hardly altered. (Unfortunately, there is an apparent contradiction in the report of Iversen, Salt and Wilson (1972); at one point they claim (Table 2) that SKF-550 at a concentration of 50 μM produced only 80% inhibition of extraneuronal uptake, whereas in Fig. 1 95% inhibition is apparently produced by only 0.3 μM SKF-550. In their discussion, these authors state that SKF-550 is, in effect, 1400 times more active in inhibiting extraneuronal uptake as neuronal uptake, which suggests a misprint in Table 2 of their paper.)

A feature of extraneuronal uptake of NA in general appears to be that it is followed by a rapid enzymic degradation, largely by O-methylation, a phenomenon which is particularly noticeable at low concentrations (Eisenfeld *et al.*, 1967a; Langer, 1970; Hughes, 1972). This finding, together with the higher specificity of extraneuronal uptake for adrenaline over noradrenaline, may be the clue to the function of the extraneuronal uptake system. In addition to playing a role in the termination of action of NA released from nerve endings but not recaptured by them, it is conceivable that extraneuronal uptake is responsible, in part at least, for the inactivation of catecholamines released into the circulation from the adrenal medulla.

✓

TABLE 1. POTENCY OF EXTRANEURONAL UPTAKE INHIBITORS

	IC50 for NA Uptake (M)		
Compound	Neuronal	Extraneuronal	Selectivity Ratio*
Phenoxybenzamine	9×10^{-7}	2.82×10^{-6}	0.3
SKF-550	4×10^{-5}	8×10^{-8}	500
17-β-oestradiol	3.7×10^{-5}	2×10^{-6}	20
Corticosterone	3×10^{-5}	2.7×10^{-6}	10

* Selectivity Ratio = IC50 Neuronal/IC50 Extraneuronal.

SYMPATHECTOMY

One way of testing the hypothesis that KCl releases sympathetic transmitter from sympathetic nerves is to remove the sympathetic nervous system from the organ or tissue being studied. In many organs and tissues, this is easily and conveniently effected by surgical means. However, some organs have such complex sympathetic innervations that surgical methods are impractical. This is exemplified in the heart, which receives sympathetic fibres from several sources. In order to produce an adequate surgical sympathectomy it is necessary to perform a cardiac transplantation, which, although not technically impossible, is exceedingly inconvenient, particularly if large numbers of animals are to be used (Goodall and Kirshner, 1956; Hertting and Schiefthalter, 1964; Cooper, Gilbert, Bloodwell and Crout, 1961; Cooper, Willman, Jellinek and Hanlon, 1962; Wegmann, Chiba, Chrysohou and Bing, 1962; Potter *et al.*, 1965; Cooper, 1966).

The alternatives to surgery, although all "chemical" in one sense may be classified in three groups:- immunological, chemical and "pharmacological".

(a) *Immunosympathectomy*

Bueker (1948) found that the implantation of a mouse sarcoma into a chick embryo caused a proliferation of sensory nerve fibres in and around the tumour. Levi-Montalcini (1952) showed further that sympathetic ganglia, as well as sensory ganglia, were stimulated by these tumors. A diffusible nerve growth factor was isolated and by chance, it was found that snake venom also contained a potent nerve growth factor (Cohen, Levi-Montalcini and Hamburger, 1954). As snake venom is produced by modified salivary glands, they examined extracts of mouse salivary glands and found that these too contained a nerve growth factor (Cohen, 1958; 1959; 1960; Levi-Montalcini and Cohen, 1956; 1960). The factor was found to be a protein and Cohen (1960) was able to produce a specific antiserum to nerve growth factor by injecting the factor into rabbits. This antiserum was found to destroy (or inhibit the development of) the sympathetic nervous system in newborn mice and rats (Levi-Montalcini and Booker, 1960; Levi-Montalcini and Cohen, 1960).

The primary effect appears to be on the paravertebral ganglia, which fail to grow, the cell counts in ganglia from treated animals being only some 2-10% of that in normal adults. The NA content of organs such as the heart and iris is hardly detectable and the dense fluorescent network which can normally be visuallized is lacking (Iversen *et al.*, 1966; Levi-Montalcini and Angeletti, 1966; Hamberger, Levi-Montalcini, Norberg and Sjöqvist, 1965) whilst the uptake of ^{3}H-NA by organs is similarly impaired (Iversen, 1965; Zaimis *et al.*, 1965; Iversen *et al.*, 1966).

The problems with this approach to sympathectomy are that one needs an established breeding colony of rats in order to treat the new born animals, which is an expensive proposition and one which requires a great deal of attention. Secondly, either a supplier of anti-nerve-growth-factor is required or a colony of rabbits together with a laboratory with facilities for blood fractionation, both of which may be prohibitively expensive for small scale experiments.

(b) Chemical Sympathectomy

In the course of an investigation into the pharmacological properties of a series of dopamine derivatives, it was found that the 6-hydroxy substituted analogue (3,4,6-trihydroxyphenylethylamine, 6-hydroxydopamine, 6-OHDA) produced a very efficient and long lasting depletion of NA from sympathetically innervated organs (Porter, Totaro and Stone, 1963; Stone, Stavorski, Ludden, Wenger, Ross, Totaro and Porter, 1963; Laverty, Sharman and Vogt, 1965). Fluorescence microscopy of tissues from 6-OHDA pretreated animals shows a complete disappearance of the terminal nerve plexus (Malmfors and Sachs, 1968; Cottle and Nash, 1974). Nerve trunks, however, are visible and have an increased intensity of fluorescence, suggesting that the cell bodies are undamaged and continue to transport storage vesicles down the axons, where they pile up at the axon stump (Geffen and Ostberg, 1969; Cottle and Nash, 1974). Electron microscopy reveals adrenergic terminals in various stages of degeneration, many being completely lysed, with the damaged fragments apparently engulfed by Schwann cells. There is no detectable change in the Schwann cells themselves, smooth muscle cells or cholinergic nerves (Tranzer and Thoenen, 1967b; 1968a,b; Thoenen and Tranzer, 1968). This highly specific destruction of noradrenergic terminals appears to be a threshold effect, as after intravenous doses of 1 mg/Kg, the NA content of rat hearts was found to be diminished to about 65% after 2 hours but to be restored essentially to normal after 24 hours. Following a dose of 3 mg/Kg I.V., however, the NA content at 2 hours was again reduced to about 65% but after 8 days, it had fallen further to about 50% of normal, whereas a dose of 30 mg/Kg produced a fall to less than 20% of normal within 2 hours, which was not restored at all over the next 8 days (Thoenen and Tranzer, 1968). After 4 weeks, however, there is a significant recovery and after 3 months, sympathetic transmitter stores are effectively normal and this is accompanied by the reappearance of newly formed adrenergic nerve endings, which apparently form by collateral sprouting from the remaining axon stumps (Tranzer and Thoenen, 1968a; Haeusler, Thoenen and Haefely, 1968a; Haeusler, Haefely and Thoenen, 1969; Hill, Mark, Eränko, Eränko and Burnstock, 1973).

The mechanism of action of 6-OHDA is unclear, although uptake at the nerve membrane but not the storage vesicle membrane is a prerequisite for nerve destruction (Stone, Porter, Stavorski, Ludden and Totaro, 1964; Malmfors and Sachs, 1968; Thoenen, 1970; Thoenen, 1972). Thoenen (1972) has suggested that 6-OHDA acts by being oxidized to the para-quinone and thence to indolines and indoles or polymerized to melanin-like macromolecules which undergo covalent binding to biologically important structures with nucleophillic groups, such as -SH, $-NH_2$ and phenolic -OH (Fig. 1).

(c) *"Pharmacological" Sympathectomy*

There are two groups of drugs which may be considered as capable of interfering with the function of sympathetic nerves to an extent sufficient to mimic sympathectomy. These are the adrenergic neurone blockers, *e.g.*, TM10, bretylium and guanethidine and secondly, drugs related to reserpine.

The modes of action of drugs classed as adrenergic neurone blockers appear to be varied and in any case, their precise actions are unknown. Reserpine, however, has a fairly well understood mechanism of action.

Reserpine causes a long lasting blockade of transmission between noradrenergic nerves and the end organs they innervate which is accompanied by a marked depletion of the transmitter content of these nerves (Holzbauer and Vogt, 1956; Carlsson, Rosengren, Bertler and Nilsson, 1957; Muscholl and Vogt, 1958). Early work suggested that these actions of reserpine were due to a blockade of the membrane uptake process (Muscholl, 1960; Axelrod, Whitby and Hertting, 1961; Hertting, Axelrod and Whitby, 1961b; Dengler, Spiegel and Titus, 1961; Brodie and Beaven, 1963). However, Kopin, Hertting and Gordon (1962) measured the initial rates of uptake of NA by hearts from normal and reserpinized rats and found no difference although the retention of NA was considerably reduced in the treated group. Similar conclusions were reached by

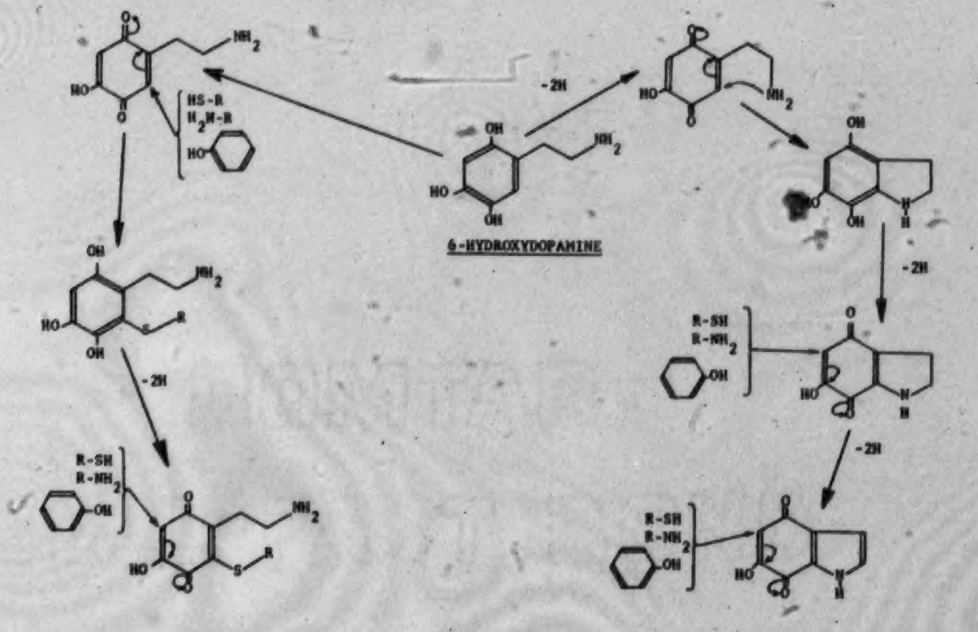

NH2
HO
HS-R
H2N-R
HO
-2H
OH
NH2
HO
OH
6-HYDROXYDOPAMINE
NH2
HO
OH
NH2
HO
OH
S
R
-2H
R-SH
R-NH2
OH
OH
H
OH
-2H
R-SH
R-NH2
OH
O
NH2
HO
S
R
O
HO
N
H
-2H
R-SH
R-NH2
OH
O
HO
N
H

Fig. 1. Possible mechanisms for the production of active derivatives of 6-OHDA. This Figure shows how chemically reactive moeities may be formed from 6-OHDA and how these may react with tissue components containing such radicles as -SH, $-NH_2$ and phenolic -OH (Thoenen, 1972).

Lindmar and Muscholl (1964) and by Iversen, Glowinski and Axelrod (1965) who also showed that most of the NA taken up by reserpinized tissues was rapidly lost in the form of deaminated metabolites.

However, when the effect of reserpine on isolated storage vesicles was investigated, it was found that low concentrations (10^{-7} M) of the alkaloid produced marked inhibition of the uptake of NA (Mirkin, Giarman and Friedman, 1963; Potter and Axelrod, 1963c; von Euler and Lishajko, 1964; Stjärne, 1964). The marked depletion of NA that occurs after reserpine, it may be surmised, is due to the failure of storage vesicles to take up NA which has been taken up into the cytoplasm by the membrane uptake mechanism. Similarly, during nerve activity, stored transmitter that is released cannot be replaced either by uptake or by synthesis from dopamine as the uptake of this precursor into vesicles is also blocked by reserpine (Stjärne, 1964). This hypothesis is supported by the finding that the rate of depletion of NA in tissues of reserpinized animals is dependent on the activity in the noradrenergic innervation of the tissue (Kärki, Paasonen and Vanhakartano, 1959; Weiner, Perkins and Sidman, 1962; Benmiloud and von Euler, 1963).

MECHANISMS OF NA RELEASE

The possible mechanisms by which NA may be released from sympathetic nerves may be summarized as follows:-

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(i) depolarization rendering the axon membrane more permeable to NA which diffuses rapidly out of the cell from a free cytoplasmic stores which is replenished from the vesicles by an equilibrium diffusion process.

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(ii) transmission of the releasing stimulus to the vesicles where the storage complex is induced to break down or the vesicle membrane is disrupted or the vesicular uptake pump is inhibited, the liberated NA then diffusing down its concentration gradient and into the synaptic cleft.

(b) Release from vesicles directly into the synaptic cleft by

(i) fusion of vesicles and axon membrane to form "tight-junctions" across which the vesicular contents pass into the cleft

(ii) extrusion of intact vesicles into the cleft

(iii) by fusion of vesicles with the axon membrane, followed by a breaking down of the composite membrane at the site of fusion so that the vesicular contents are extruded into the cleft. This possibility has been termed "reverse pinocytosis" or "exocytosis".

(a) Release via the Cytoplasm

The possibilities listed above under this heading require that NA released from vesicles into the cytoplasm shall diffuse to the synaptic cleft intact. That this is an unlikely proposition is demonstrated by experiments employing reserpine, which inhibits vesicular uptake of NA without altering the axon membrane uptake process (Kopin *et al.*, 1962; Kirshner, 1962; Carlsson *et al.*, 1963; Stjärne, 1964; Iversen *et al.*, 1965). In reserpinized tissues, ^{3}H-NA is taken up at the same initial velocity as normal, but the ^{3}H-label cannot be recovered as NA but only as metabolites, almost exclusively deaminated products, indicating that NA is by no means immune to enzymic degradation when in free solution in the cytoplasm (Iversen *et al.*, 1965). By treating with both reserpine and inhibitors of MAO before exposing to exogenous NA, however, tissue levels of NA can be made to equal or exceed the levels in untreated tissues (Potter, 1967; van Orden *et al.*, 1967a; Häggendahl and Malmfors, 1969; Farnebo and Hamberger, 1971). At these high cytoplasmic levels of NA, passive efflux of NA is very much less than that occurring during normal nerve activity, indicating that simple diffusion across the axon membrane is inadequate (Potter, 1967; Löffelholz, 1973). If the nerves are stimulated, there is no apparent increase in the efflux of NA and transmission of impulses to the innervated muscle is not restored (Owman and Sjöstrand, 1966; Potter, 1967; van Orden *et al.*, 1967a; Farnebo and Hamberger, 1971). Lindmar and Löffelholz (1972) also showed the marked temperature dependence of efflux from such loaded nerves in contrast to the relative insensitivity of electrically induced release from normal nerves.

Further evidence against a mechanism involving diffusion through the cytoplasm and axon membrane is the finding that the soluble proteins of the vesicles (dopamine-β-hydroxylase and chromogranin A) can be detected in the effluent from perfused and stimulated spleens, whereas soluble cytoplasmic proteins of similar sizes or less cannot (Geffen and Livett, 1968; Geffen, Livett and Rush, 1969a; 1970b; de Potter *et al.*, 1969b; de Potter, Moerman , Schaepdryver and Smith, 1969a; Smith, de Potter, Moerman and Schaepdryver, 1970).

It has also been shown that only structural analogues of NA which are taken up by both the axon membrane pump and the vesicular pump can be released on nerve stimulation; those analogues which are taken up by the axon membrane only cannot be released (Potter, 1966).

(b) *Direct Release from Vesicles into Synaptic Cleft*

The concept of passage of transmitter across a "gap-" or "tight-junction", formed by close apposition of vesicle and plasma membranes was proposed for central synapses by Pfenniger, Akert, Moor and Sandri (1971) on the basis of freeze-etched electronmicrographs. However, on inspection, their micrographs appeared not to be incompatible with the concept of exocytosis.

According to Kanno and Loewenstein (1966) proteins of molecular diameter of 7 nm or less can cross tight junctions between cells. The molecular diminsions of dopamine-β-hydroxylase and chromogranin A, however, are of the order of 12 nm (Smith, 1973), so that it is unlikely that this mechanism operates, as these proteins are released along with NA as described above.

The possibility of extrusion of whole vesicles has been investigated using the insoluble vesicle membrane-bound form of dopamine-β-hydroxylase as a marker. Analysis of the effluent from perfused and stimulated spleens revealed only the soluble form of this protein, in addition to which, prolonged stimulation failed to reduce the tissue content of the membrane-bound form (Smith *et al.*, 1970; Chubb, de Potter and Schaepdryver, 1972; Smith, 1973).

Strong support for the concept of exocytosis, as proposed by de Robertis and vas Ferreira (1957a,b) for release of catecholamines is found in the adrenal medulla:-

(a) Release of catecholamines from perfused and stimulated adrenals is accompanied by the same stoichiometric proportions of nucleotides and soluble proteins as are found in isolated vesicle preparations (Banks and Helle, 1965; Douglas and Poisner, 1966; Kirshner, Sage, Smith and Kirshner, 1966; Blaschko, Comline, Schneider, Silver and Smith, 1967; Sage, Smith and Kirshner, 1967; Schneider *et al.*, 1967; Trifaro and Poisner, 1967).

(b) Lipid components of vesicle membranes (phospholipids and cholesterol) are not released on stimulation and there is no decrease in the lipid content of vesicle preparations made from depleted glands (Poisner, Trifaro and Douglas, 1967; Schneider *et al.*, 1967; Trifaro, Poisner and Douglas, 1967).

(c) Typical cytoplasmic proteins, such as lactate dehydrogenase and DOPA-decarboxylase, are not released with vesicle contents (Schneider *et al.*, 1967; Viveros *et al.*, 1968).

(d) Electron micrographs apparently showing fusion of vesicles with the plasma membrane and the actual exocytosis have been published (de Robertis and vas Ferreira, 1957a,b; Coupland, 1965; Diner, 1967; Fillenz, 1971).

In the case of noradrenergic nerves, the evidence in favour of exocytosis is less clear. Perhaps the best, though tenuous, evidence is that noradrenergic nerves bear a close embryological and functional relationship to adrenal medullary cells. Unlike the neuromuscular junction of striated muscle, the distance between smooth muscle cells and the noradrenergic fibres which innervate them is very variable and is in any case considerably larger - 20 to 1000 nm (Burnstock, 1970). Consequently the concentration of NA arriving at the post-synaptic membrane depends upon both the quantity of NA released and the distance it has had to diffuse and is therefore liable to vary. The demonstration of spontaneous junction potentials (SJP) from smooth muscle cells with noradrenergic innervations (Burnstock and Holman, 1962; 1966; Orloff, 1963) parallels the demonstration of miniature end plate potentials (MEPP) at neuromuscular junctions of skeletal muscle (Fatt and Katz,

1952) except that the amplitudes of SJPs appear to be continuously graded, in contrast to the discontinuous distribution seen with MEPPs. The gradation of SJPs could be due to either variation in the size of transmitter quanta or to the differences in the distances between release sites and post-junctional activation sites (Holman, 1970).

Several workers have tried to calculate the amount of NA released per varicosity during nervous activity (Folkow, Häggendahl and Lisander, 1967; Folkow and Häggendahl, 1970; Bevan, Chesher and Su, 1969; Bell and Vogt, 1971) and these estimates range from about 400 to 9000 molecules of NA per varicosity per stimulus. These workers also suggest that each vesicle contains about 10,000 - 15,000 molecules of NA, so that it would appear that either 2.5% to 60% of the content of single vesicles are released per varicosity per stimulus or that the total content of one vesicle is released from between 2.5% and 60% of the varicosities per stimulus (Smith and Winkler, 1972). This latter is also consistent with the view that the contents of a single vesicle are released from each varicosity every 2-40 stimuli, which correlates well with the finding that in the sympathetic supply to the pulmonary artery, a response is not obtained until a critical number of pulses has passed (6-8) (Bevan *et al.,* 1969). As it is conceivable that many varicosities are "within range" of any smooth muscle cell, then the varying distances could explain the variable SJP size.

The demonstration of chromogranin A and dopamine-β-hydroxylase in the effluent from perfused spleens and the absence of cytoplasmic enzymes clearly supports the applicability of the exocytosis concept to adrenergic nerves, although the stoichiometry is less well established than it is for adrenal medulla (Geffen *et al.,* 1969a; de Potter *et al.,* 1969b; Smith *et al.,* 1970).

The electronmicroscopic evidence for exocytosis from noradrenergic nerves is scanty, at best, although Fillenz (1971) has published micrographs apparently showing fusion of vesicles with axon membranes and one of her micrographs shows what may be an opening between the inside of a vesicle and the extracellular space. Another of her micrographs shows an apparently empty vesicle still attached to the plasma membrane, although Dr. Fillenz states that "Fusion of the vesicle membranes with the axon membrane is only rarely seen in nerve terminals from normal tissues". An interesting speculation regarding the rarity of such events in electron micrographs would be that as fixation probably progresses from the axon membrane inwards, and if the fusion of vesicles with the axon membrane follows the random bombardment of the latter with vesicles in Brownian motion, then as the viscosity of the axoplasm near the membrane increases with the inward spread of fixation, the probability of finding a vesicle there goes down. Only those vesicles actually trapped by fusion with the axon membrane will be seen with any regularity and the possibility of dissociation being induced as the fixation reaches the outer layers of membrane remains (*i.e.*, an "allosteric" dissociation of vesicles and axon membrane).

In a combined electronmicroscopic, pharmacological and functional study, van Orden *et al.* (1967a) found that treatment with reserpine produced a progressive degranulation of vesicles, which could be correlated with the reduction in NA content of the tissue and impairment of neuromuscular transmission.

It therefore seems quite likely that exocytosis is the mechanism by which NA is released from sympathetic nerves, but until the stoichiometry and biochemistry of the process are more fully understood, it must remain a hypothesis only.

ELECTROPHYSIOLOGY OF NORADRENERGIC NERVES

Because of their size, intracellular recordings from peripheral noradrenergic nerves are not feasible except in ganglia where the cell bodies are large enough to allow penetration with micro-electrodes, although here, too, penetration is not easy owing to the tough connective tissue sheaths around ganglion cells. However, extracellular recordings are possible, either by surface electrodes (Eccles, 1935) or by the sucrose-gap method (Kosterlitz and Wallis, 1966) as well as extracellular recording using micro-electrodes (Skok, 1968) although this latter is rare.

The electrophysiological study of terminal noradrenergic fibres is fraught with difficulties and is essentially only possible indirectly, either by recording anti-dromically from the non-terminal axon or by recording post-junctional potentials from the innervated muscle cells (Burnstock and Holman, 1960).

The membrane potential of the sympathetic ganglion cells has been found to be between -40 and -70 mV (Haefely, 1972). According to Haefely (1972) these estimates are more likely to be low than high and consequently the true, "typical" resting membrane potential of a ganglion cell is likely to be nearer -70 mV than -40 mV. In frog sympathetic para-vertebral ganglion cells (intracellular electrodes) and rabbit superior cervical ganglia (sucrose gap) the membrane potential was found to be proportional to the logarithm of the external K^+ concentration ($[K^+]_o$) for $[K^+]_o$ above 8 - 10 mM, reduction of $[K^+]_o$ below this having little effect on membrane potential (Blackman, Ginsborg and Ray, 1963; Kosterlitz, Lees and Wallis, 1968). The reason for the resting membrane potentials being less than the K^+ equilibrium potential is not clear, although in the intracellular study (Blackman *et al.*, 1963) changes in the leakage resistance of the microelectrodes could explain this difficulty. Blackman *et al.* (1963) also showed that in frog ganglion cells, sodium and chloride potentials do not contribute measurably to the membrane potential.

Historically, it was Bernstein (1902) who realized that the thermodynamic principles described by Nernst and Ostwald could be applied to electrophysiology. The Nernst equation was originally derived to describe the electrochemical equilibrium between the electrical work needed to move a small quantity of ions across a boundary and the osmotic work needed to move the same number of ions in the opposite direction. Bernstein postulated that the membrane of excitable cells was selectively permeable to K^+ alone when at rest but that during excitation, numerous "pores" opened, allowing penetration by other small ions, primarily Na^+ and Cl^-. This theory explained the maintenance of the high intracellular K^+ and low intracellular Na^+ as being due to the inability of Na^+ to move down its concentration gradient because of the impermeant membrane, the leakage of K^+ being opposed by the development of an electrostatic force. The membrane potential generated by the charge separation was then described by the Nernst equation:-

$$E = \frac{RT}{F} \ln \frac{[K^+]_{in}}{[K^+]_{out}}$$

The theory accurately predicts that the potential will fall as the external $[K^+]$ is raised.

However, although the Nernst equation can be applied to excitable cells, the reason for the ionic separation has been found not to fit Bernstein's postulates. It was found, instead, that both Na^+ and K^+ are in a steady state of flux across the membrane and at comparable rates (Levi and Ussing, 1948; Harris and Burn, 1949; Keynes, 1954). The maintenance of the electrochemical gradients necessary for maintaining the membrane potential were finally found to be due to the existence of the so-called "Sodium Pump". In this system, a basal level of metabolic energy is utilized to transport Na^+ ions out of the cell at a rate equivalent to the rate at which they leak in, simultaneously transporting K^+ ions into the cell, Na^+ and K^+ apparently being exchanged on a one-for-one basis (Hodgkin and Keynes, 1955). Thus, the development of a potential is due to the much higher permeability of the membrane to K^+ than to Na^+.

The excitable cell membrane can be represented by the equivalent electrical circuit diagram shown in Fig. 2.

The actual value of the membrane potential will be somewhere between the extremes of the equilibrium potentials for Na^+ and K^+, being closer to the channel with the predominant conductance. In fact, it is invariably found that the membrane potential is much closer to E_K than E_{Na} as the permeability and conductance of the membrane at rest to Na^+ are much lower than to K^+.

If we consider what change will be expected in the membrane potential when the external KCl concentration is raised, we will find that the influences of the two ionic species, K^+ and Cl^- are in opposite direction. That is to say, elevating the $[K^+]_{out}$ will tend to make the membrane potential less negative (*i.e.*, depolarization) whereas high $[Cl^-]_{out}$ will tend to hyperpolarize the membrane. If just K^+ is taken into account, in the ideal situation the membrane potential change will be inversely proportional to the logarithm of $[K^+]_{out}$:-

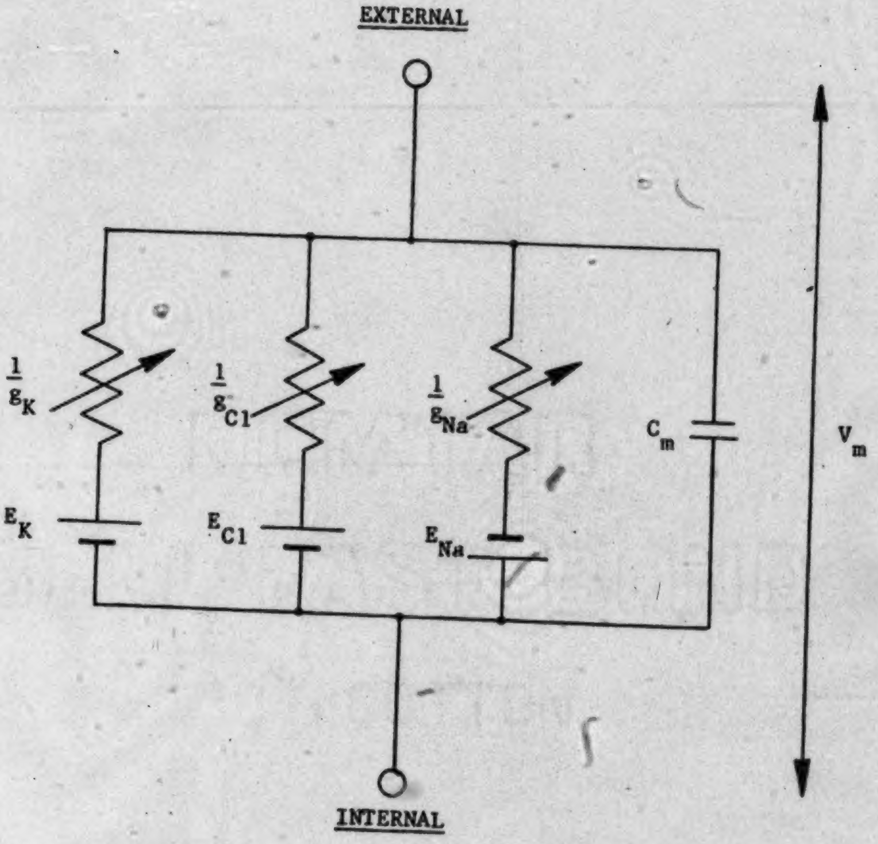
EXTERNAL
$\frac{1}{g_K}$
$\frac{1}{g_{Cl}}$
$\frac{1}{g_{Na}}$
C_m
V_m
E_K
E_{Cl}
E_{Na}
INTERNAL

Fig. 2. Equivalent circuit diagram for cell membrane.

V_m = membrane potential

g_i = conductance of membrane to ionic species i

E_K = equilibrium potential of K^+ (-70 to -100 mV)

E_{Na} = equilibrium potential of Na^+ (+50 to +65 mV)

E_{Cl} = equilibrium potential of Cl^- (-45 to -90 mV)

Inside negative

$$E = \frac{RT}{F} \ln \frac{[K^+]_{out}}{[K^+]_{in}}$$

However, as mentioned above, this relationship was found to apply when $[K^+]_{out}$ exceeded 8 - 10 mM (Blackman *et al.*, 1963).

Hodgkin and Horowicz (1959) were able to fit the experimental points obtained from frog skeletal fibres to an equation taking into account a contribution from Na^+:-

$$V_{K^+\alpha Na} = \frac{RT}{F} \ln \frac{[K^+]_{out} + \alpha[Na^+]_{out}}{[K^+]_{in} + \alpha[Na^+]_{in}}$$

where α is defined as the permeability ratio, P_{Na}/P_K (Hodgkin and Katz, 1949). In this instance the value of α was 0.01. In their paper, Hodgkin and Horowicz (1959) point out that the predicted relationship between membrane potential and $[K^+]_{out}$ only applies when equilibrium is reached. In order to change the values of either $[K^+]_{out}$ or $[Cl^-]_{out}$ without disturbing the equilibrium, therefore, they accompanied changes in the (external) concentration of one of these ions with a reciprocal change in the concentration of the other, so that the product $[K^+]_{out} \cdot [Cl^-]_{out}$ remained constant (Boyle and Conway, 1941). This they achieved by replacing a suitable proportion of the anion with the methylsulphate ion ($-CH_3SO_4^-$). In this way rapid (instantaneous) and reversible changes in membrane potential may be produced without inducing any net fluxes of K^+ or Cl^- across the cell membrane.

Kosterlitz, Lees and Wallis (1967) recording from an isolated superior cervical ganglion of a rabbit by the sucrose gap technique, obtained results similar to Hodgkin and Horowicz (1959) when they raised $[K^+]_{out}$ either by substituting KCl for NaCl or by adding K_2SO_4 and keeping the $[K^+]_{out} \cdot [Cl^-]_{out}$ product constant, although they do not report how long the ganglion was allowed to remain in the test solution before the potentials were measured. Blackman *et al.* (1963) measured the membrane potential in frog sympathetic ganglia using micro-electrodes and found that the potential changes induced by the addition of isotonic KCl were not obviously different from those obtained when Cl^- free Ringer's solution was used (substituted with $-CH_3SO_4^-$). They concluded that the contribution of Cl^- ions to the membrane conductance was small, although their attempts to fit the measured resting potential and external $[K^+]$ to the Nernst equation or the modified equation of Hodgkin and Horowicz were not particularly successful. However, their finding that changes in potential with $[K^+]_{out}$ were essentially the same when either Cl^- or $-CH_3SO_4^-$ was the external anion strongly suggests that the contribution of Cl^- to the membrane conductance was vanishingly small. They supported this conclusion by showing that replacement of Cl^- by $CH_3SO_4^-$ did not produce the transient depolarization predicted if there was a significant Cl^- channel in the membrane. Whether this holds true for mammalian sympathetic neurones is uncertain, although the work of Kosterlitz *et al.* (1967) tends to support this possibility. However, if there is an appreciable Cl^- channel, then elevation of $[Cl^-]_{out}$ will tend to oppose the depolarization produced by high $[K^+]_{out}$.

MECHANISM OF THE TRANSMITTER-RELEASING ACTION OF KCl

(a) *Ionic Basis of Transmitter Release*

Much of the work aimed at elucidating the ionic events which occur in the nerve terminal during the transduction of the action potential to the release of transmitter has been carried out on cholinergic systems, largely because of the ease with which post-synaptic electrical activity can be recorded. Using the frequency of m.e.p.p.s as an indication of the amount of transmitter (ACh) released, it has been found that manouevers which depolarize the pre-synaptic nerve induce transmitter release in proportion to the magnitude of the depolarization, irrespective of the manner in which the nerves are depolarized (Del Castillo and Katz, 1954; Liley, 1956; Hagiwara and Tasaki, 1958; Takeuchi and Takeuchi, 1962; Lester, 1970). That the release is due to depolarization *per se* and not to changes in Na^+ and K^+ conductance associated with the action potential is shown by the findings that transmitter release could be ellicited in response to direct depolarization following treatment with tetrodotoxin (TTX), which abolishes the propagated action potential by selectively preventing the increase in Na^+ conductance upon which this process depends (Katz and Miledi, 1967). Similarly, tetraethylammonium (TEA), which prevents the development of an outward K^+ current, does not prevent ACh release when depolarization is induced by current injection from an internal electrode (Katz and Miledi, 1969). However, removal of Ca^{++} from the bathing medium results in a failure of ACh release, although the action potential persists (Dodge and Rahamimoff, 1967; Katz and Miledi, 1969).

The essential nature of external Ca^{++} ions in transmitter release has been formalized in the "calcium hypothesis" which states, in essence, that during depolarization of the terminal part of the axon, *i.e.*, that part from which transmitter release occurs, the normally Ca^{++} impermeant membrane (P_K/P_{Ca} = 1000; Hodgkin and Keynes, 1957) becomes more permeable to Ca^{++} ions and that the resultant influx of Ca^{++} ions in turn initiates or controls transmitter release (Katz and Miledi, 1965). Katz and Miledi (1967) obtained evidence which strongly supports the argument that Ca^{++} influx is dependant upon the membrane potential. They used the giant synapse of the squid which they treated with TTX and TEA in order to block both the Na^+ and K^+ channels and thus prevent propagated activity. The synapse was prepared with a micro-electrode in the pre-synaptic fibre for stimulation and a recording micro-electrode in both the pre- and post-synaptic fibres. By applying stimuli of relatively long duration (to all intents and purposes voltage-clamping the pre-synaptic fibre) and by recording from the post-synaptic fibre for a number of increasingly large stimuli, they found that the post-synaptic response, *i.e.*, transmitter release, increased but then fell off at higher potentials until it completely disappeared, whilst a response now appeared when the stimulus was switched off. In this paper (Katz and Miledi, 1967) and in another (Katz and Miledi, 1969) these authors obtained values for this "Suppression potential" of between +70 mV and +140 mV. In other words the equilibrium potential for the ion responsible for transmitter release is between +70 and +140 mV, this ion presumably being Ca^{++}. The surge of transmitter release which occurred during the repolarization of the pre-synaptic fibre after depolarizations beyond this equilibrium potential can then be explained as being due to an influx of Ca^{++} during the time that the membrane is recovering its resting potential, assuming that the permeability of the membrane to Ca^{++} is largely determined by the membrane potential. In addition, it might be expected that during prolonged depolarizations in excess of the Ca^{++} equilibrium potential Ca^{++} ions would move down their electrochemical gradient out of the nerve cell, so that during repolarization there could be an enhanced influx of Ca^{++} due to an increased inwardly directed concentration gradient. The major stumbling block to the immediate acceptance of this evidence as conclusive support for the "calcium hypothesis" is that a knowledge of the free, ionized, intracellular Ca^{++} concentration is necessary to allow calculation of the Ca^{++} equilibrium potential and although this is not currently available as an incontravertible fact, estimates are available which would put the calcium equilibrium potential well within the range of the "suppression potential" described above (Hodgkin and Keynes, 1957; Katz and Miledi, 1969; Miles, 1969). Further evidence that ACh secretion depends upon an influx of Ca^{++} was provided by Blaustein (1971), who showed an increased uptake of ^{45}Ca during ACh release induced by nerve stimulation.

In adrenergic nerves the position seems similar, in that NA release depends upon depolarization itself and that the action potential with its attendant Na^{+} and K^{+} shifts is unnecessary, whilst external Ca^{++} ions are again obligatory requirements. Thus in a number of sympathetically innervated organ preparations, removal of Ca^{++} ions from the bathing solution has been found to inhibit NA release (Kirpekar and Misu, 1967; Haeusler *et al.*, 1968a, b; Kirpekar and Wakade, 1968; Bisby and Fillenz, 1969; Nash *et al.*, 1972). Release of adrenaline from the adrenal medulla also requires Ca^{++} (Douglas and Rubin, 1963).

Perhaps the most relevant work to the problem being considered here has been performed by Haeusler *et al.*, using the perfused, innervated, cat heart. By placing recording electrodes on the sympathetic nerves to the heart, and perfusing the coronary circulation with solutions containing ACh and/or high K^+, they were able to record anti-dromic nervous activity (Haeusler *et al.*, 1968 a, b; Haeusler, Haefely and Huerlimann, 1969). They found that K^+ caused a brief burst of anti-dromic action potentials and that ACh caused a more sustained firing. When ACh was infused into the heart in the presence of high K^+ and after the initial burst of firing in response to the K^+ had subsided, they were unable to obtain the usual sustained action potential activity, which demonstrates in an indirect manner that high K^+ was causing a sustained depolarization. They were also able to demonstrate that both ACh and high K^+ caused a release of NA from the heart and that this release was unaffected by TTX which abolished the propagated electrical activity normally associated with these agents. They also demonstrated the essential nature of Ca^{++} for the release of NA in two ways:

1. Perfusion of the hearts with solutions free of Ca^{++} prevented the ACh and K^+ induced release of NA without altering the recorded electrical activity.
2. Inclusion of amethocaine (tetracaine) in the perfusing fluid abolished the induced release of NA before the electrical activity was impaired. (Amethocaine has been shown to inhibit Ca^{++} influx without altering Na^+ flux [Douglas and Kanno, 1967].)

Such evidence has led Haefely (1972) to conclude that "....electrosecretory coupling at adrenergic nerve endings seems....to be essentially similar to that previously found at motor nerve endings....and in the adrenal medulla....". In other words, the stimulus for transmitter release is an influx of Ca^{++} ions which is itself the result of a permissive alteration in membrane permeability associated with depolarization.

As stated at the beginning of this introduction, the use of elevated K^+ has been used by many workers as a stimulus for transmitter release, the universal assumption being that its action in this regard is mediated solely by a depolarization of the nerves in the tissue under study. In the adrenergic system, there would appear to be only one study which in any way answers the question of whether this is in fact the mode of action of K^+ and this evidence was discussed above (Haeusler *et al.*, 1968a). Thus although the studies on NA release mechanisms which have relied upon K^+ stimulation are important in our understanding of the subject, the basic premise that the K^+ induced release of NA is qualitatively comparable with that evoked by electrical stimulation of the sympathetic nerves remains untested (Haeusler *et al.*, 1968a; Starke, 1972a, b; Starke and Montel, 1974).

In this context, it is interesting to note that there are two reported instances in which there is a difference between K^+ induced NA release and that induced by some other, but yet nerve stimulating, method. In one (Starke and Montel, 1974) α-adrenoceptor mediated feed-back inhibition of K^+ induced NA release was apparently less effective than when release was induced with dimethylphenylpiperazinium (DMPP). In the other, (Stjärne, 1973) it was found that K^+ induced release of NA was apparently not accompanied by a release of prostaglandin E as was that induced by nerve stimulation.

Thus, although it can undoubtedly be argued that high K^+ initiates release of NA from adrenergic nerves by virtue of its nerve depolarizing action, one is not able to argue the complementary point, that K^+ produces no release of NA from any other sites or by any other mechanism.

(b) *Exocytosis vs. Diffusion*

Although Burnstock and Holman (1966) demonstrated an increase in the frequency of spontaneous junction potentials (S.J.P.s) following electrical stimulation of the adrenergic nerves to vasa deferentia, such an experiment does not appear to have been performed using high K^+ as the stimulus (*cf.* Liley, 1956). However, if the assumptions of Haefely (1972) given above are correct, and if normal, physiological NA release occurs by exocytosis, then perhaps it is reasonable to conclude that as K^+ produces depolarizations of sympathetic ganglion cells which agree tolerably well with those predicted by the Nernst equation (Blackman *et al.*, 1963; Kosterlitz *et al.*, 1967), then its mode of action in releasing NA from nerves is by exocytosis. Such a conclusion is supported by the findings that both electrically induced NA release and that induced by high K^+ are inhibited by the removal of Ca^{++} from the bathing or perfusing solution or the addition of Mg^{++} to it (Kirpekar and Misu, 1967; Boullin, 1967; Haeusler *et al.*, 1968a, b; Kirpekar and Wakade, 1968; Bisby and Fillenz, 1969; Nash *et al.*, 1972). On the other hand, NA release induced by the so-called "indirectly acting sympathomimetics" (Trendelenburg, 1963) is independent of Ca^{++} (Lindmar, Löffelholtz and Muscholl, 1967; Haeusler *et al.*, 1968).

STATEMENT OF THE PROBLEM AND EXPERIMENTAL APPROACH

Given that elevated KCl causes a release of ^{3}H-NA from labelled, perfused rat hearts, there are a number of questions which must be asked concerning the mechanisms underlying this phenomenon.

(a) Is the release caused by the KCl or is it osmotically induced?

(b) Does the Cl^- contribute to the phenomenon or is it solely due to the K^+ ion?

(c) In what way is release of ^{3}H-NA related to $[K^+]$?

(d) From what site (neuronal or extraneuronal) does the release occur and by what mechanism?

(a) KCl or Osmotic Effect?

This question may be resolved by comparing the effects of KCl and equiosmolar concentrations of both an electrolyte and a non-electrolyte, *e.g.*, choline chloride and sucrose.

(b) K^+ or Cl^-?

The effect of choline chloride from (a) will in part answer this question, the use of a potassium salt other than a halide (*e.g.*, potassium methylsulphate) complementing this.

(c) Relation Between $[K^+]$ and Release

This requires a determination of the dose/response curve of $[K^+]$ *vs.* NA release.

(d) *Site(s) and Mechanism(s) of Release*

The first step in this part of the investigation would be to determine the part played by uptake into and release from the adrenergic nervous supply of the heart by achieving some form or forms of sympathectomy. The second step would then be to examine the effects of inhibitors which are reported to be specific or selective for the various sites of potential uptake and release.

METHODS

HEART PERFUSION

Male Wistar rats of 150 - 250 g were killed by cervical dislocation and the hearts removed into a dish of cooled physiological salt solution where blood was removed from the chambers by gentle squeezing. The aorta was cannulated and perfused at a constant rate of 4 ml/min by means of a peristaltic pump (Harvard Apparatus Co., Inc.) using the apparatus shown in Fig. 3. The perfusing solution was a Krebs-bicarbonate solution of the following composition (mM):- NaCl, 118.5; KCl, 4.8; $CaCl_2$, 2.5; KH_2PO_4, 1.4; $MgSO_4$, 1.2; $NaHCO_3$, 24.9; dextrose, 9.1; disodium ethylenediaminetetracetic acid (Na_2EDTA), 0.03; ascorbic acid, 0.06, or a modification of this as described below. It was gassed with oxygen containing 5% carbon dioxide and was maintained at 37°C. The solution perfusing the hearts could be rapidly changed by means of two stop-cocks. Effluent was continuously collected in test tubes on a fraction collector set to 2 min per tube.

The hearts were perfused for 10 min with the normal Krebs solution before any infusions were begun and any hearts that were not beating well at the end of this period were assumed to have compromised circulations and were discarded. This was a rare occurrence.

Modifications to Perfusing Solutions

(a) High K^+, Na^+ and Li^+ solutions.

KCl, NaCl, LiCl, potassium methyl sulphate or sodium methyl sulphate (KCH_3SO_4, $NaCH_3SO_4$, Electronic grade, City Chemical Corporation, New York) was added to the normal solution.

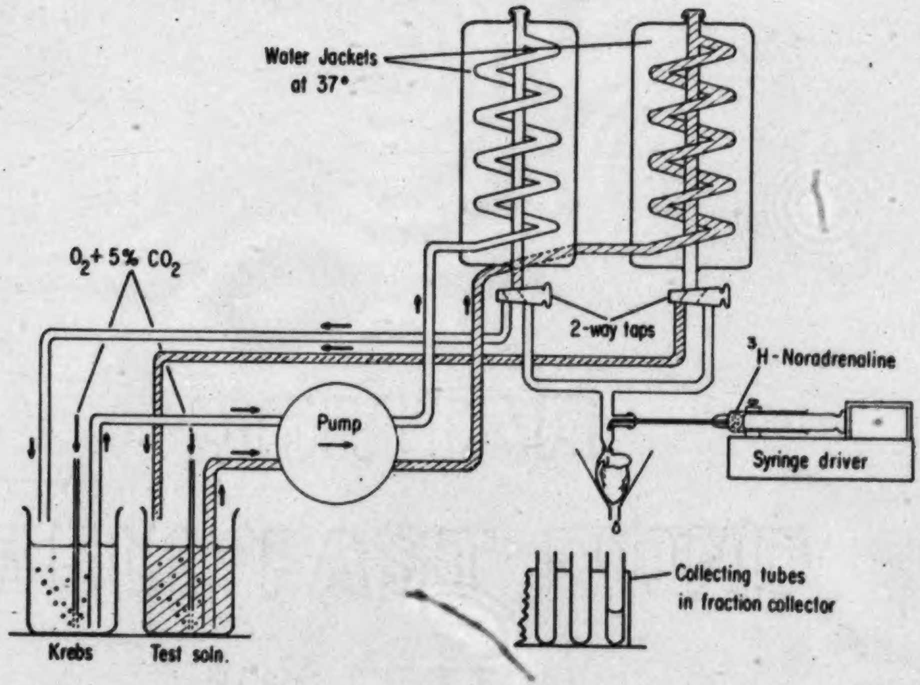
Water Jackets at 37°
O_2+ 5% CO_2
2-way taps
^{3}H-Noradrenaline
Pump
Syringe driver
Collecting tubes in fraction collector
Krebs
Test soln.

Fig. 3. Heart perfusion apparatus.

Unless otherwise specified, the reported concentration of these high K^+ and Na^+ solutions in the text refers to the excess of cation. Thus, the solution referred to as 56 mM K^+ in reality contained a total of 62.2 mM K^+. These solutions were hypertonic.

(b) High sucrose and choline chloride.

These solutions were prepared as osmotic comparisons with 56 mM KCl Krebs. It was assumed that a solution containing 56 mM choline chloride would have the same osmolarity as 56 mM KCl Krebs or very close to this. The concentration of sucrose needed to make a solution equally hyperosmotic, however, is not immediately obvious. The osmolarity of 56 mM KCl Krebs was therefore determined using an osmometer (Advanced Instruments, Inc.).

A series of 3 concentrations of sucrose in Krebs was also prepared (75 mM, 100 mM and 112 mM) and the osmolarity of these solutions was determined in the same way. The sucrose concentration which was equally hyperosmolar with 56 mM KCl Krebs was then determined by interpolation on a graph of sucrose molarity *vs.* osmolarity (Fig. 4). This concentration of sucrose was found to be 90 mM. The osmolarity of 56 mM choline chloride Krebs was also measured and found to be 392 mOsm compared with 386 mOsm for 56 mM KCl.

(c) K^+ free solution.

This was the same as the normal Krebs solution except that the KCl was replaced with NaCl and the KH_2PO_4 was omitted.

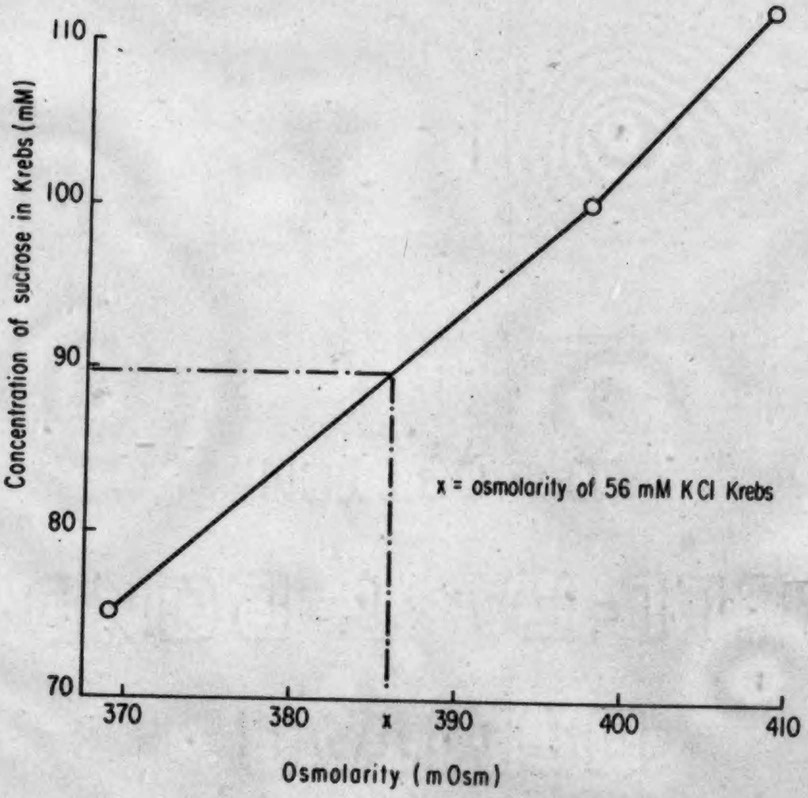

Fig. 4. Effect of sucrose concentration on osmolarity of Krebs solution. This was used to determine the concentration of sucrose needed to make a Krebs solution with the same osmolarity as 56 mM KCl Krebs.

(d) Perfusion solutions containing drugs.

These were made up to the reported concentrations immediately before the first experiment of the day by serial dilutions of stock solutions.

(e) Loading of transmitter stores.

Tritiated NA (ℓ-NA-7-^{3}H; New England Nuclear) with a specific activity of 6.41 or 3.7 Ci/mM was made up to a 2×10^{-5} M stock solution in 0.01 N HCl containing 0.1 mM Na_2EDTA and 0.06 mM ascorbic acid and stored at 4°C in the dark. For infusion, this stock solution was diluted to 4×10^{-6} M with 0.01 N HCl containing 0.1 mM Na_2EDTA, 0.06 mM ascorbic acid and 154 mM NaCl. This solution was injected into the perfusion solution just above the aortic valve of the heart by means of a narrow, polyethylene tube attached to a motor-driven syringe (Sage Instruments, Inc.). The injection rate was 0.1 ml/min in all experiments. This rate gave a final concentration of 10^{-7} M NA in the perfusion solution. For efflux experiments, the ^{3}H-NA was injected for 10 min, after which time the effluent was collected as described above.

For uptake experiments, the concentration of the ^{3}H-NA was adjusted so that a 0.1 ml/min injection rate gave the desired concentration, and the infusion time was adjusted so that it was within the linear portion of the uptake *vs.* time curve (see "Results").

UPTAKE STUDIES

A series of experiments was performed to determine the effect of high concentrations of K^+ on the initial rate of uptake of ^{3}H-NA. ^{3}H-NA was infused as described earlier at a suitable concentration for a suitable time (see "Results") after which the heart was removed, blotted dry, frozen in liquid nitrogen and extracted as described later and samples taken for radioactivity determinations. To correct for radioactivity trapped in the vessels and extracellular space of the heart, estimates of this volume were made using ^{14}C-mannitol. The ^{14}C-mannitol was infused at a concentration of 6×10^{-7} M (20.8 Ci/mol) for 1, 2, 4 or 8 min in:-

(a) normal hearts,

(b) hearts which were being simultaneously infused with unlabelled NA, 5×10^{-7} M,

(c) hearts which had been infused for 4 min with 112 mM KCl Krebs and which were similarly perfused throughout the mannitol infusion period.

The hearts were then removed and extracted as described below and the radioactivities determined, from which values for the ^{14}C-mannitol tissue water spaces were determined (volume/weight %). From these a correction term for the ^{3}H-NA uptake was determined.

When uptake of ^{3}H-NA was to be determined in the presence of altered concentrations of K^+, the hearts were perfused with the test solution for 4 min before the ^{3}H-NA infusion was commenced.

DETERMINATION OF RADIOACTIVITY

Radioactivity was determined by liquid scintillation spectrometry using a Beckmann LS-230 liquid scintillation counter, corrections for quench being made by the channels ratio method. Fig. 5 shows a standard curve of counting efficiency *vs*. channels ratio using a set of six sealed tritiated toluene samples (Beckman Instruments, Inc.) with varying amounts of quench. A standard curve such as that shown in Fig. 5 was prepared every second month; no appreciable change was detected over a 2 year period.

The composition of the toluene based fluor was as follows:- toluene, 1 litre; 2,5-diphenyloxazole (PPO), 4.606 g; 1,4-Bis-(5-phenyloxazol-2-yl)-benzene (POPOP), 0.115 g; "Biosolve" (BBS-3, Beckmann), 0.153 litres. This cocktail was found to give good counting efficiency when counting aqueous samples constituting up to 12% by volume of the total (Fig. 6). As this liquid scintillation counting was performed using Mini-vials (Nuclear Associates, Inc.) which will hold up to 6 ml, the standard counting procedure was to add 0.5 ml of aqueous sample to 4.5 ml of fluor, shake thoroughly and count. It can be seen from Fig. 6 that these proportions (*i.e.*, 10% aqueous sample) fall on a relatively flat part of the curve, giving counting efficiencies of better than 40%. Dark adaptation was found to produce no discernable advantage. When the radioactivity of heart extracts (see later) containing ^{14}C was required, a technique similar to the ^{3}H counting technique was used. Interpolation on the ^{14}C-standard curve of counting efficiency *vs*. channels ratio (Fig. 7) gave counting efficiencies in excess of 90% (usually *ca*. 93%).

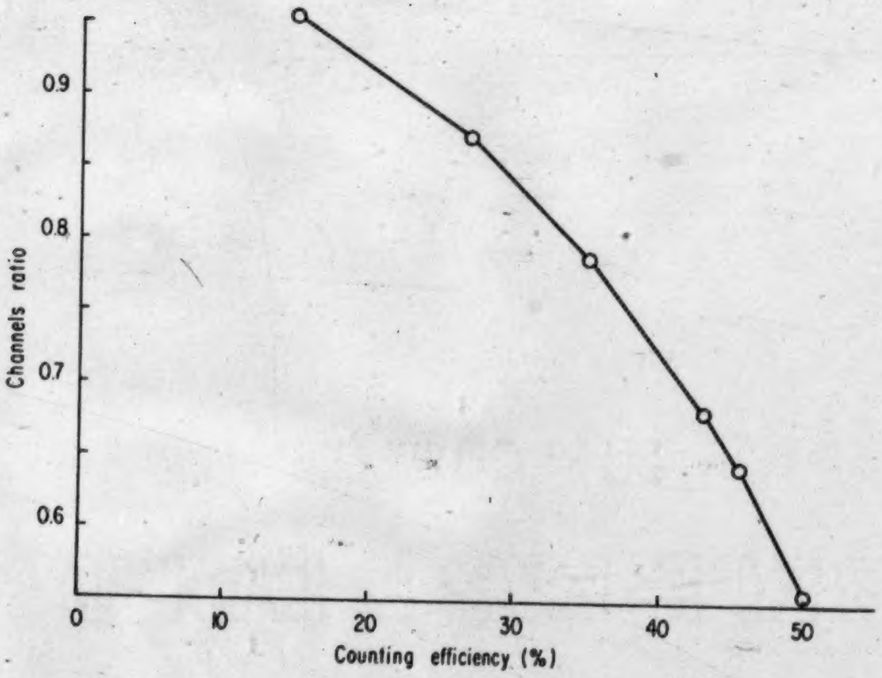

Fig. 5. Quench correction curve for ^{3}H.

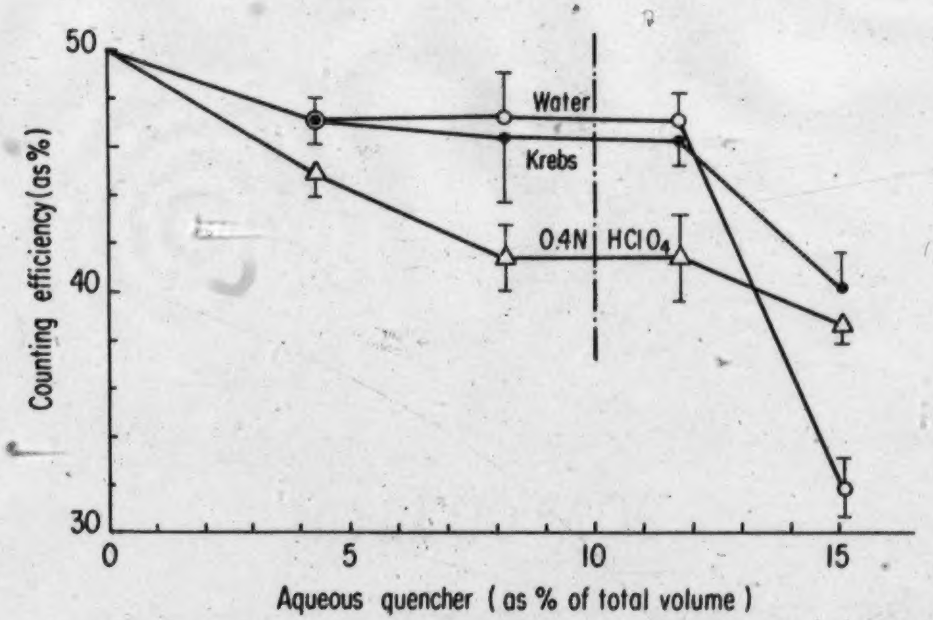

Fig. 6. Effect of sample volume on counting efficiency. Means ± S.E.M.; n = 4. The vertical line (— . — . — . —) indicates the sample volume used in subsequent experiments. The data for this Figure are given in the Appendix, Table 3.

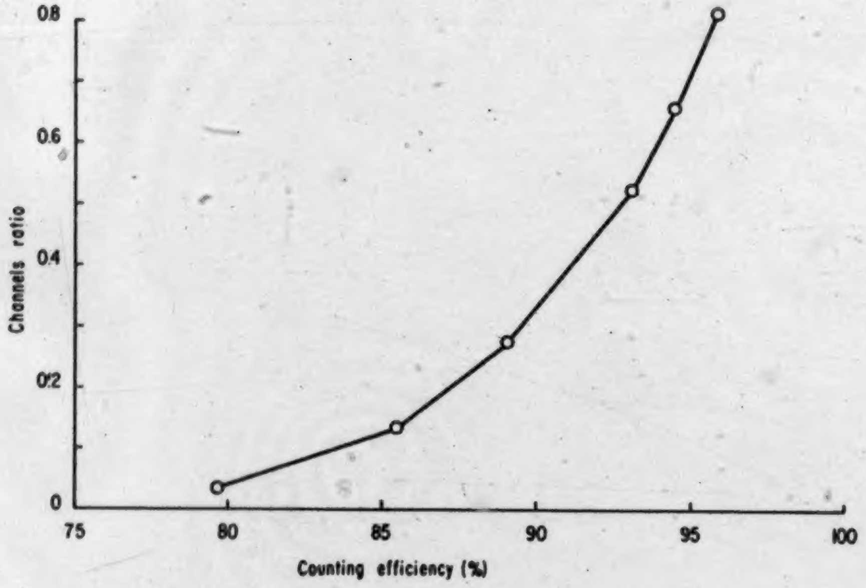

Fig. 7. Quench correction curve for ^{14}C.

Determination of Radioactivity in Heart Tissue

At the end of both efflux experiments and uptake experiments the heart was removed from the cannula and cut open by two bold scissor cuts from the apex towards the base, the plane of each cut being perpendicular to the other. The heart was then blotted dry on absorbent paper, frozen in liquid nitrogen and stored in a covered beaker at -10°C until extracted. The storage period was kept as short as possible. The heart was then weighed, cooled down in liquid nitrogen and pulverized by concussion in a stainless steel die (Fig. 8), the powder/liquid nitrogen slurry poured into a Duall glass homogenizer tube (Size C, Kontes Glass Company) and 4 volumes of 0.4 N perchloric acid containing 0.01 M Na_2EDTA added. Much of the added solution then froze to a mush, but with agitation on a vortex mixer (Vortex-Genie, Fisher Scientific Co.) most of the particles could be readily dispersed through the soft ice. This was then homogenized using a motor driven Duall type ground glass pestle. To attempt to introduce some degree of standardization into this procedure, the homogenization was accomplished by using 20 complete strokes. The homogenate was then allowed to stand for 10 min before centrifuging at 12,000 x g_{AVE} at 4°C in an angle head rotor. Quadruplicate 0.5 ml samples of the supernatant were removed and their radioactivities determined by liquid scintillation counting as described above and their means were determined. (By re-homogenizing and re-centrifuging the pellet twice more, it was found that a further 5 to 10% of radioactivity could be extracted. This further extraction was

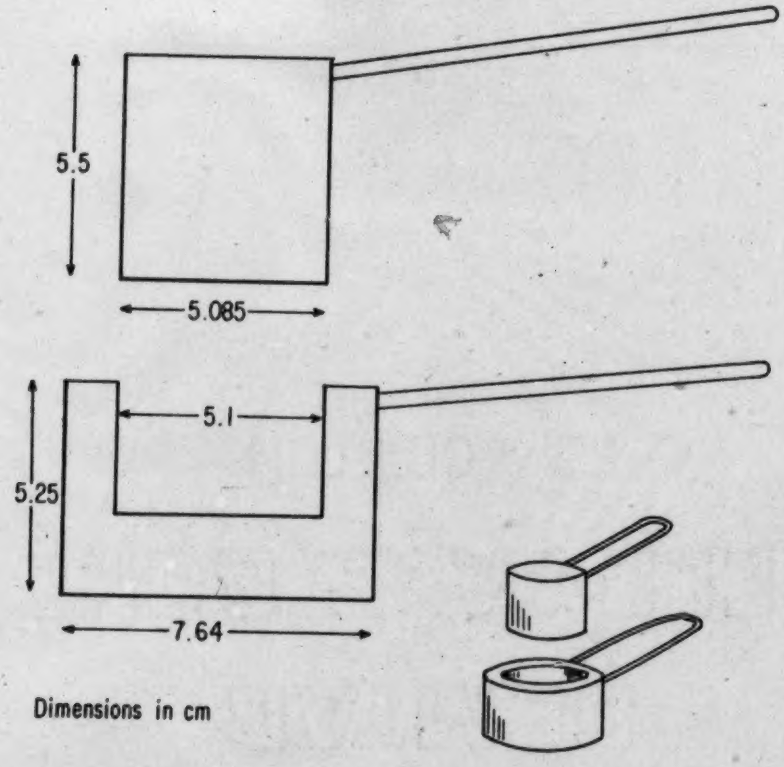
5.5
5.085
5.1
5.25
7.64
Dimensions in cm

Fig. 8. Stainless steel die used to pulverize hearts.

not done in these experiments and the activities obtained were not corrected for this.)

CALCULATION OF RESULTS

(a) *Efflux Experiments*

The results of the liquid scintillation counting were collected on punched paper tape and processed with either a PDP-8 mini-computer (Digital Equipment Corporation) equipped with a Teletype punched-tape reader or with the central University computing facility (IBM 360/67) using MTS-FORTRAN. The two programs and an explanation are given in the Appendix. In essence, the programs converted the raw counts to disintegrations by means of linear interpolation on a standard curve. From this and other keyboard input of variables, three values were calculated for each sample and printed in three columns. The first column is the efflux from the heart in disintegrations/min/g heart weight/min. The second column is the total heart content of radioactivity at that time, calculated by back-addition of the efflux values to the activity remaining in the heart at the end of the experiment. The third column comprises efflux coefficients or efflux rate constants, which are the fractional rates of loss per unit time:-

$$\text{Efflux Coefficient} = \frac{\Delta A}{\Delta t . A_t}$$

where, ΔA = the dpm lost in the time interval Δt and A_t is the amount of radioactivity in the heart at the mid-point of the interval Δt (Hopkin and Neal, 1971). However, as effects could not be compared directly when efflux coefficients were used , all values reported in "Results" are based on absolute efflux. In some experiments, effluents for each

30 min were pooled and quadruplicate 0.5 ml aliquots removed for radioactivity determinations. In this way, total efflux could be calculated for each 30 min period, which when combined with a value for the tissue content at the end of the experiment, yielded values for total tissue ^{3}H content at the beginning of the efflux period and after 30, 60, 90 and 120 minutes of efflux.

Induced release of ^{3}H, I, was determined by adding the efflux values (corrected for time) for the times corresponding to the test period (60 - 90 min). This term, ^{3}H-release, is expressed in pmol/g/test period.

$$I = \sum_{t=60}^{t=90} \frac{E.t_c}{s} \text{ pmol/g}$$

where, E = efflux (dpm/g/min)

t_c= collecting time for each sample

s = specific activity (dpm/pmol).

In experiments where it was necessary to compare the effects of pretreatment, 3 parameters were chosen for comparison. These were:-

(a) Total uptake of ^{3}H-NA. This was the first value in the second column of the computer printout. It is referred to as Heart Content$_{t=0}$, *i.e.*, at zero efflux time.

(b) Retention of ^{3}H-NA. This value is the last value before the test period in the second computer printout column and is referred to as Heart Content$_{t=60}$, *i.e.*, after 60 min efflux.

(c) Release of ^{3}H. This was calculated as described above.

In all cases Content$_{t=0}$ and Content$_{t=60}$ are expressed as % of control, *i.e.*, as percentages of the ^{3}H-NA contents at either t=0 or t=60 of hearts from all animals which were not pretreated (121 animals). In some cases, release was also expressed as percentage of control. In this case the control release value was the amount of radioactivity released from normal hearts by a 30 min challenge with 56 mM KCl.

(b) *Uptake Studies*

When determining uptake, it is necessary to make a correction for radioactivity remaining in the extracellular space at the end of the uptake period and to subtract this from the total uptake of radioactivity. This was done by determining a correction factor comprising the extracellular space, radioactivity in the perfusing solution and the weight of the heart.

The extracellular fluid volume of the hearts at time t expressed as a percentage of the weight of the heart was calculated according to the formula:-

$$V_t = \frac{D \times R}{A} \times 100$$

where, D = radioactivity (^{14}C) in sample of heart extract (dpm)
R = dilution factor = 5/sample volume = 5/0.5 = 10
A = activity of ^{14}C-mannitol (dpm/ml).

The value of 5 used in the determination of R derives from the use of 4 volumes of homogenizing fluid, *i.e.*, 4 volumes + 1 volume of heart (assuming the specific gravity of heart tissue to be 1.0).

The initial velocity of ^{3}H-NA uptake, v, was calculated according to the formula:-

$$v = \frac{(B \times R) - C}{Q \times t} \text{ pmol/g/min}$$

where, B = radioactivity in heart extract sample (dpm)

R = dilution factor (usually 10)

C = correction term

$$= \frac{V_t \times W \times S}{100}$$

W = heart weight (g)

S = activity of infused ^{3}H-NA (dpm/ml)

V_t = extracellular space (%)

Q = specific activity of infused ^{3}H-NA (dpm/pmol)

t = uptake time.

Tests of significance were made by the analysis of variance technique, the F-test and comparison of means method described by Sokal and Rohlf (1969). Differences were deemed significant when $P<0.05$.

PURITY OF ^{3}H-NA

The purity of the ^{3}H-NA was periodically checked by cellulose thin layer chromatography using a modification of the method of Fleming and Clark (1970). The ^{3}H-NA solution was spotted onto plastic-backed cellulose TLC sheets (Eastman #6064) and run in a solvent composed of 1-butanol:methanol:1N formic acid (3:1:1). The chromatograph was then cut transversely into 1 cm strips and the cellulose scraped off into scintillation vials where it was mixed with toluene fluor and sequentially counted. In some cases, the whole strips were placed in the vials, covered with fluor and counted. The counts were plotted against distance from the origin and RF values for the peak(s) determined. These values were then compared with the RF value obtained from the chromatograph of unlabelled standard NA which had been sprayed with a colour developer (diazotized p-nitroaniline) sensitive to catecholamines and their derivatives. The procedure for this was as follows:-

Solution A = 0.1 g p-nitroaniline (Eastman Co.) was dissolved in 2 ml concentrated HCl and diluted to 100 ml with water. This solution was kept at 4°C.

Solution B = 0.2 g $NaNO_2$ was dissolved in 100 ml water. This solution was kept in a dark bottle at 4°C for no more than one month.

Solution C = 20 g K_2CO_3 was dissolved in 200 ml water.

Equal volumes of solutions A and B were mixed and allowed to stand for 10 min on ice and then 2 volumes of solution C were added and mixed. The mixture was sprayed onto the plates in a fine spray using an atomizer (Kontes Glass Co.) until the plates were damp and translucent but not visibly wet. The coloured spots develop in a few minutes and are quite stable. It was possible to detect less than 1 nmole of NA by this colour reaction.

The radioactivity in chromatographs of ^{3}H-NA was always confined to a single narrow band, the RF of which corresponded to the RF for NA obtained using the colour development method. Any purity that was present was not detectable above the background (see Fig. 9 for a typical radio-chromatograph).

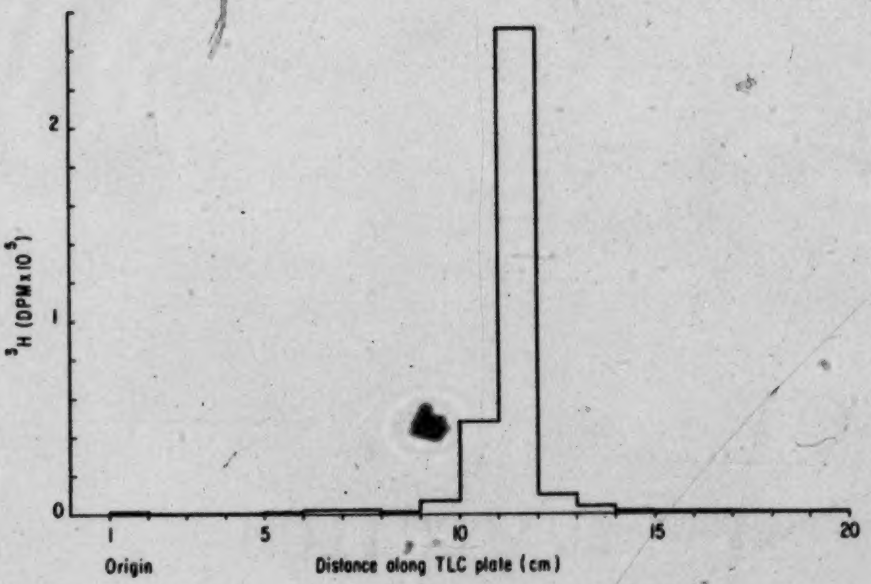

Fig. 9. Radiochromatogram of stock ^{3}H-NA. Means of 4 spots, each of 10 μℓ ^{3}H-NA, 4 mM (6.41 mCi/μM).

DENERVATION

(a) *Chemical Sympathectomy*

Thoenen (1972) has suggested that the optimal treatment schedule for sympathectomy of rats is two doses of 6-OHDA 50 mg/Kg I/V given within 24 hours, followed by two further doses of 100 mg/Kg given a week later. In another study, however, Cottle and Nash (1974) found that a repeated dose of 6-OHDA was no more effective than a single dose in causing degeneration of peripheral adrenergic plexi. In their study, these latter authors used 100 mg/Kg 6-OHDA given intraperitoneally, which may result in a slower and more prolonged liberation into the blood stream than occurs after intravenous injection.

Consequently rats were treated with a single dose of 6-OHDA, 100 mg/Kg by intraperitoneal injection. The drug (6-OHDA.HBr) was dissolved in 20% ascorbic acid immediately before injection. After 36-48 hours the hearts were removed and perfused as described above.

(b) *"Pharmacological" Sympathectomy*

Rats were treated with reserpine 2 mg/Kg by intraperitoneal injection and after 18 - 24 hours with a second dose of 1 mg/Kg given by the same route. The reserpine was kept as a 1 mg/ml solution in 20% ascorbic acid in brown glass bottles. The hearts were removed and perfused one hour after the second dose of reserpine.

MATERIALS

NaCl, KCl, $MgSO_4.7H_2O$, $CaCl_2$, $NaHCO_3$, Na_2HPO_4, dextrose, 1-butanol, K_2CO_3 and $NaNO_2$ (A.C.S. grade) were obtained from Fisher Scientific Co.; hydrochloric acid (A.C.S.) from Baker Chemical Co.; p-nitroaniline and choline chloride from Eastman Organic Chemicals; Cellulose T.L.C. plates (6064) from Eastman Kodak Co.; sucrose, Grade I, from Sigma Chemical Co.; KCH_3SO_4 and $NaCH_3SO_4$, electronic grade, from City Chemical Corp., N.Y.; 6-hydroxydopamine.HBr from Terochem Laboratories Ltd., Edmonton; formic acid, A.C.S., from Allied Chemical Co; methanol and toluene, A.C.S., from Mallinckrodt, Canada, Ltd; PPO, scintillation grade, from Packard Instrument Co., Inc.; POPOP, scintillation grade, from Aldrich Chemical Co. Inc.; "Bio-Solv" BBS-3 from Beckman Instruments Inc.; ℓ-NA-7-^{3}H and ^{14}C-D-mannitol from New England Nuclear.

I wish to acknowledge gifts of DMI and reserpine from CIBA-Geigy Ltd., and SKF-550 from Smith, Kline and French, Ltd.

RESULTS

OSMOTIC AND IONIC EFFECTS

Fig. 10 shows the way in which the efflux of ^3H changes with time. It can be seen that after about 45 - 50 min, the curve is approximately log linear. As this suggests that only a single compartment is contributing to the efflux at this time, treatments with KCl or other substances were not begun until efflux had been going on for 60 min.

When the perfusing solution was changed to Krebs containing an excess of 56 mM KCl, a marked increase in ^3H efflux was induced. This is shown in Fig. 11. The induced efflux reached a peak after approximately 4 min of KCl and then fell to a well-maintained plateau for the remainder of the 30 min KCl treatment before falling to approximately the same level as controls (Fig. 10) when the perfusing solution was changed back to normal.

In order to test whether this release phenomenon was due to the KCl *per se* or to the increased tonicity of the solution, the total release induced by equiosmolar sucrose (90 mM) was compared with that caused by 56 mM KCl. From Fig. 12, it can be seen that treatment with 90 mM sucrose reduced, rather than increased the efflux of ^3H, although this reduction was not significant when compared with control. A group of hearts was also exposed to 56 mM choline chloride, which resulted in an increase in the efflux of ^3H (Fig. 12) which was not significantly different from control, whilst the release induced by 56 mM KCl was very significantly different from control (P= <0.01).

In an attempt to study the effect of chloride ions further,

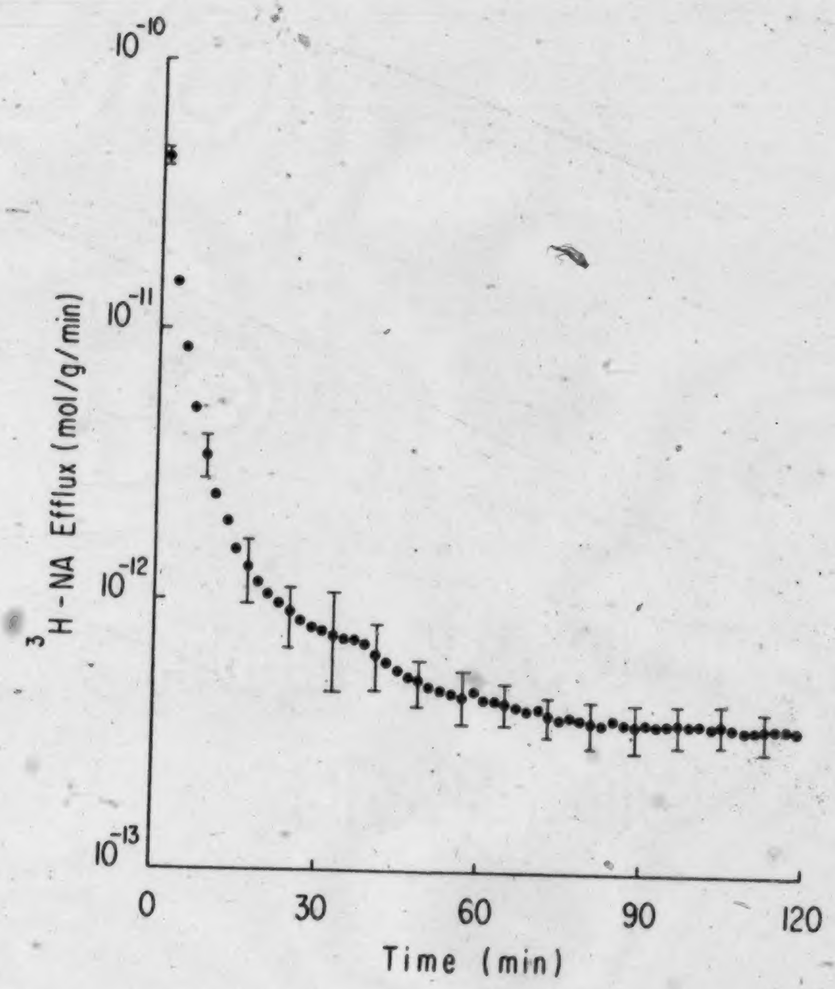

Fig. 10. Efflux of ^{3}H-NA from perfused hearts. Means ± S.E.M.; n = 6.

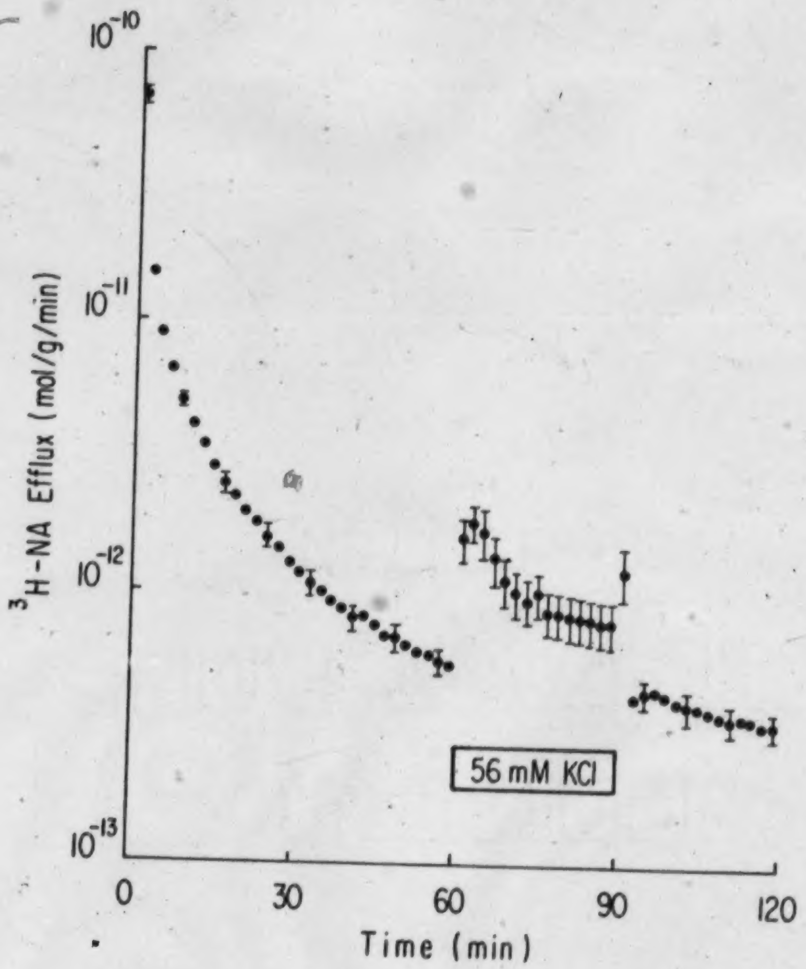

Fig. 11. Effect of 56 mM KCl on ^{3}H-NA efflux from perfused hearts. Means ± S.E.M.; n = 16.

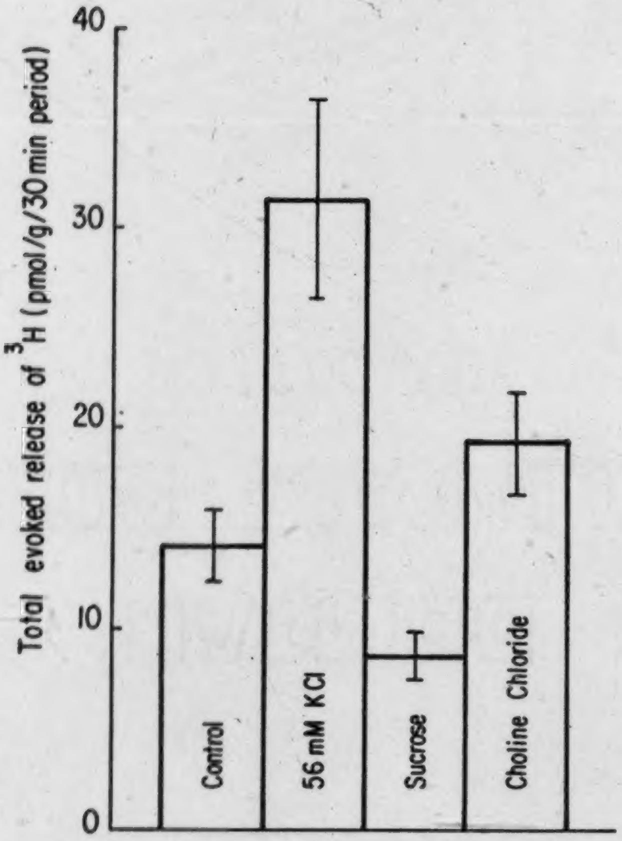

Fig. 12. Effect of osmolarity and ionic strength on ^{3}H-NA efflux from perfused hearts. Means ± S.E.M.; for control, n = 9; for KCl, n = 16; for sucrose, n = 6; for choline, n = 7. The data for this Figure are given in the ~~Appendix,~~ Table 4.

groups of hearts were exposed to 56 mM sodium chloride, sodium methyl sulphate, potassium methyl sulphate and lithium chloride. The results of these treatments are shown in Fig. 13. Treatment with NaCl, $NaCH_3SO_4$ and with KCH_3SO_4 caused increases in efflux which were not significantly different from KCl treatment. The release of ^3H induced by 56 mM LiCl was not significantly different from either control or KCl treatment.

CONCENTRATION DEPENDENCE

The release of ^3H-NA induced by concentrations of KCl between 14 mM and 224 mM in excess of that in normal Krebs solution was measured and the results are shown in Fig. 14, where total ^3H release is plotted *vs.* the log of the total external $[K^+]$. It can be seen that there was a spontaneous efflux of ^3H and that an increase in efflux did not become apparent until the total $[K^+]_{out}$ exceeded about 35 mM. As the $[K^+]_{out}$ was increased above this threshold level, however, there was a concentration dependent increase in efflux, which appeared to be proportional to log $[K^+]_{out}$.

UPTAKE STUDIES

(a) *Determination of the Extracellular Fluid Volume of the Rat Heart*

The ^{14}C-mannitol space (V_t) was determined after ^{14}C-mannitol infusions of 1, 2, 4 and 8 min in the presence of normal Krebs, 112 mM KCl Krebs and normal Krebs plus 5×10^{-7} M NA (unlabelled). The results are plotted as % water [(ml/g) x 100] *vs.* time (min) in Fig. 15. It can be seen that in the presence of 5×10^{-7} M NA, high values of V_t

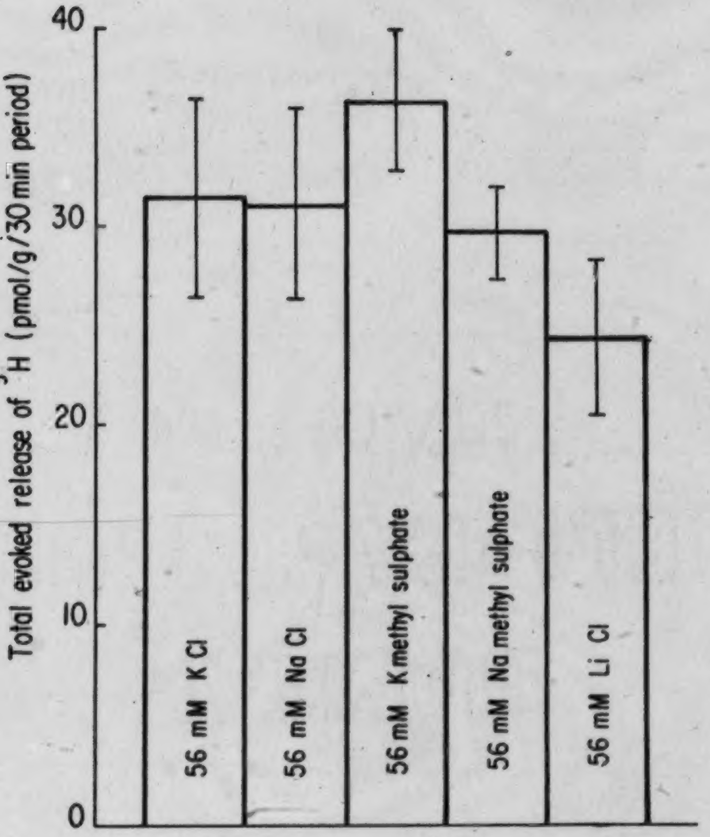

Fig. 13. Effects of anions and cations on ^{3}H-NA efflux from perfused hearts. Means ± S.E.M.; n = 5, except for KCl, where n = 16. The data for this Figure are given in the Appendix, Table 5.

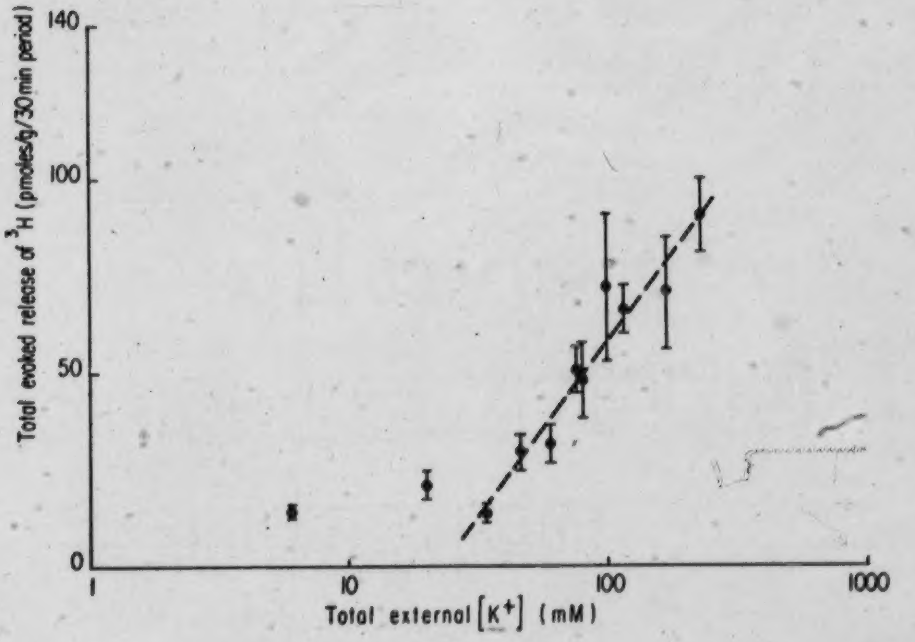

Fig. 14. Effect of K$^+$ concentration on efflux of ^{3}H-NA from perfused hearts. Mean ± S.E.M. (n = 5-16). The data for this Figure are given in the Appendix, Table 6.

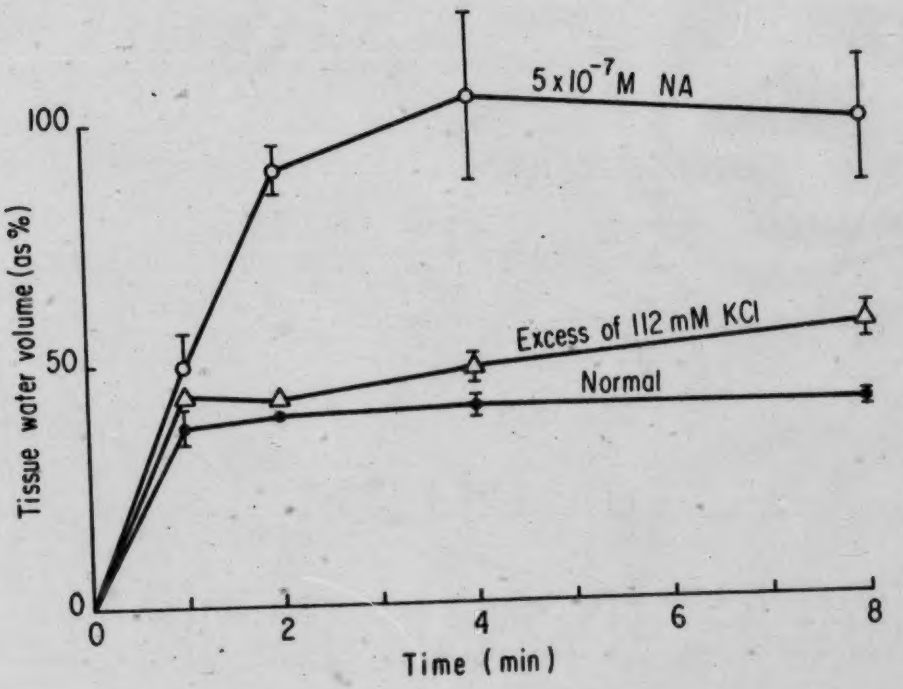

Fig. 15. Determination of extracellular space with ^{14}C-mannitol. Means ± S.E.M.; n = 5. The data for this Figure are given in the Appendix, Table 7.

were obtained, whilst V_t determined in the presence of 112 mM KCl was only slightly larger than with normal Krebs. None of these latter differences were significantly different from their corresponding controls, whereas all the values obtained in the presence of 5×10^{-7} M NA were very significantly different from their respective controls ($P < 0.01$) except that determined at t=1 min, which was not significantly different.

In all subsequent experiments, uptake of ^{3}H-NA was corrected for V_t using a value of 40% (*cf.* Nash, Gillespie and Robertson, 1974).

(b) *Uptake of ^{3}H-NA vs. Time*

Uptake of ^{3}H-NA was determined after 1, 2, 4 and 8 min infusion of 2×10^{-7} M ^{3}H-NA. The results, expressed as pmol/g are plotted *vs.* time (min) in Fig. 16. The straight line was fitted by the method of least squares; slope = 80.58,

Y intercept = 0.184.

As no discernable fall-off in the rate of uptake over the time course studied was observed, 5 min was chosen as the standard infusion time for further uptake studies. This time was within the linear range and also gave a convenient compromise between the levels of radioactivity which could be easily counted and the use of a minimum amount of costly tracer.

(c) *Effect of Zero K^+ on ^{3}H-NA Uptake*

^{3}H-NA uptake was determined in the presence of normal (6.2 mM) and zero K^+ at ^{3}H-NA concentrations of 1, 1.25, 1.67, 2.5 and 5.0 x 10^{-8} M. The results are presented as a double reciprocal plot (Line-

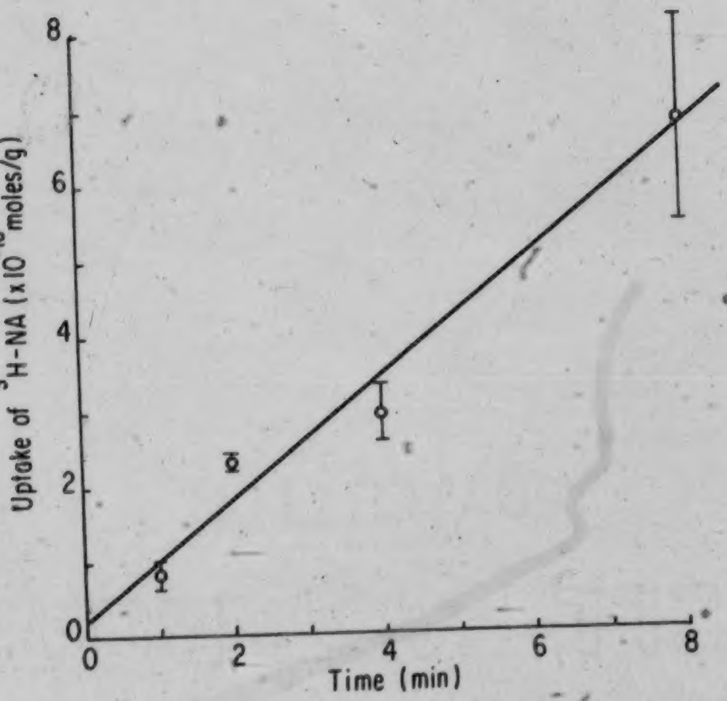

Fig. 16. Effect of time on ^{3}H-NA uptake velocity. Means ± S.E.M.; n = 5. The data for this Figure are given in the Appendix, Table 8.

weaver and Burk, 1934) in Fig. 17, in which the regression lines were determined by the least squares method.

In the absence of K^+, the values of K_m and V_{max} were 0.0944 µM and 84.36 pmol/g/min, whilst in normal K^+ they were 0.0935 µM and 120.26 pmol/g/min, respectively, indicating an alteration in the maximum rate of uptake without any change in the apparent affinity between NA and its postulated uptake carrier.

(d) *Effects of Raised K^+ on ^{3}H-NA Uptake*

Uptake of ^{3}H-NA was determined at ^{3}H-NA concentrations of 1, 1.25, 1.67, 2.5 and 5.0 x 10^{-8} M in the presence of total $[K^+]_{out}$ of 34, 62, 118 and 230 mM. The uptake velocities (mol/g/min) are plotted *vs.* NA concentration for the different K^+ concentrations, including normal (6 mM) and zero K^+ in Fig. 18.

In Fig. 19, the same results are plotted in the alternative manner; *viz.* uptake velocity *vs.* $[K^+]_{out}$ for the different ^{3}H-NA concentrations. It can be seen that in all cases, except at 5 x 10^{-8} M NA, reducing or raising the $[K^+]$ from normal reduces the uptake velocity. In the case of 5 x 10^{-8} M NA, the optimum uptake appears to occur at about 30 mM KCl.

INHIBITION OF UPTAKE

The effect of a challenge with a 30 min exposure to 10^{-7} M desmethylimipramine (DMI) was determined in a group of hearts labelled with ^{3}H-NA as described above. It can be seen (Fig. 20) that this treatment caused a large increase in the efflux of ^{3}H, which was not significantly different from the efflux induced by 56 mM KCl. When a

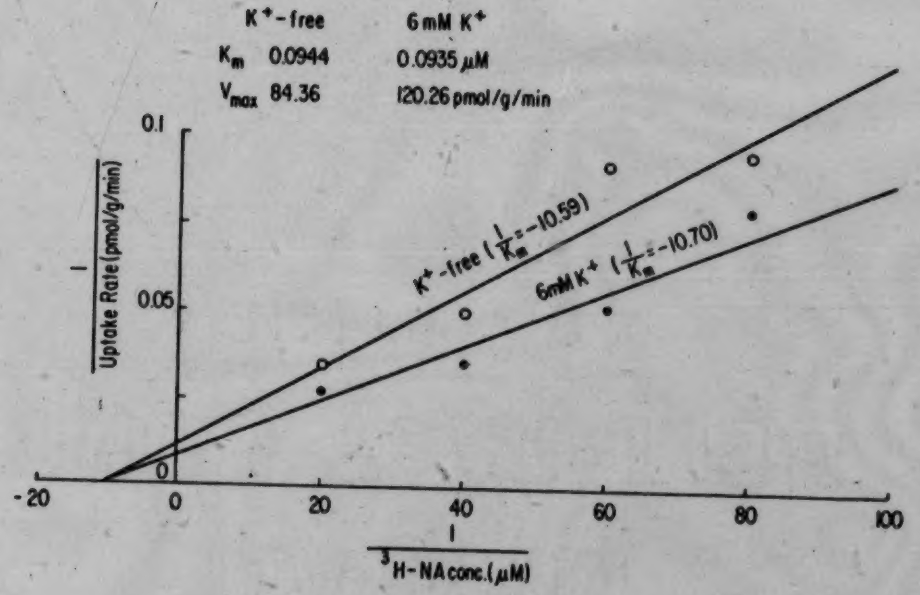

Fig. 17. Effect of zero [K^+] on ^{3}H-NA uptake kinetics. The K_m was determined from the x-intercept and V_{max} from the slope. The regression lines were fitted by the method of least squares. The data for this Figure are given in the Appendix, Table 9.

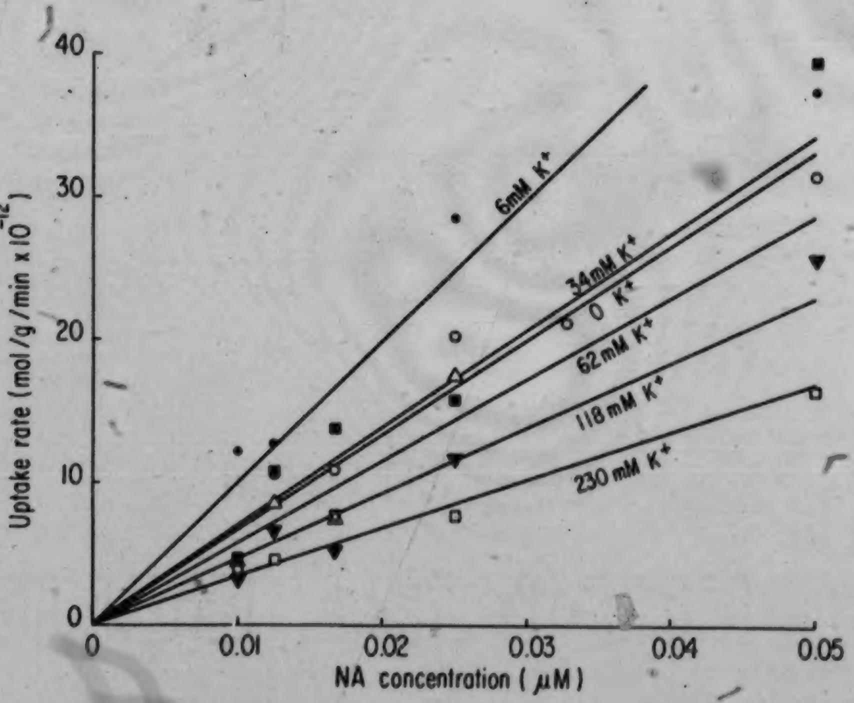

Fig. 18. Effect of $[K^+]$ on ^{3}H-NA uptake (i)

○—○ = 0 K^+

●—● = 6 mM K^+

■—■ = 36 mM K^+

△—△ = 62 mM K^+

▼—▼ = 118 mM K^+

□—□ = 230 mM K^+

The data for this Figure are given in the Appendix, Table 9.

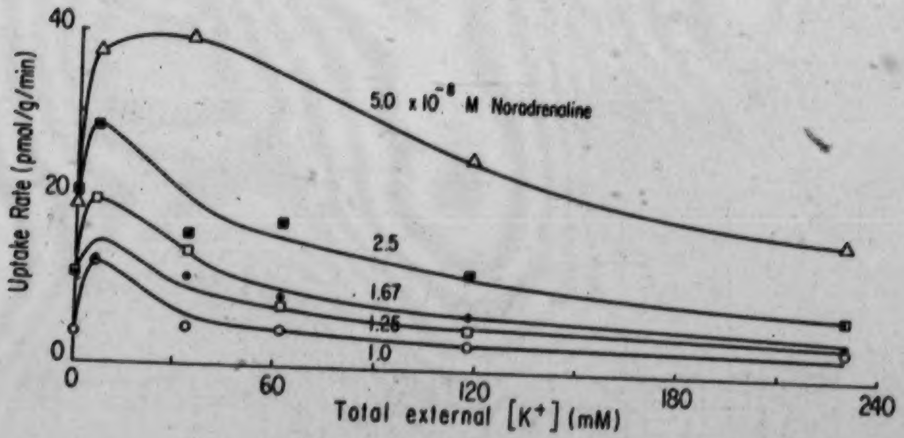

Fig. 19. Effect of [K$^+$] on ^{3}H-NA uptake (ii)

○—○ = 0.01 μM ^{3}H-NA

●—● = 0.0125 μM ^{3}H-NA

□—□ = 0.0167 μM ^{3}H-NA

■—■ = 0.025 μM ^{3}H-NA

△—△ = 0.05 μM ^{3}H-NA

The data for this Figure are given in the Appendix, Table 9.

group of hearts was treated with 10^{-7} M DMI and 56 mM KCl simultaneously, the induced efflux was not significantly different from that evoked by 56 mM KCl alone or 10^{-7} M DMI alone (Fig. 20).

When extraneuronal uptake was inhibited with SKF-550 throughout both the loading and efflux periods, uptake at t=0 was not significantly altered (see Table 2). After 60 min efflux, however, the amount of ^3H retained by hearts in the presence of SKF-550 was greatly reduced (96.58 *vs.* 351.17 pmol/g; $P < 0.01$). Passive efflux (*i.e.*, without 56 mM KCl) between t=60 and t=90 in the presence of SKF-550 was 14.85 pmol/g, compared with 14.16 pmol/g from untreated hearts (no significant difference). When release was stimulated by 56 mM KCl, the release in the presence of SKF-550 was 25.54 pmol/g compared with 31.39 pmol/g from untreated hearts, which was also an insignificant difference.

DENERVATION STUDIES

(a) *6-Hydroxydopamine*

Pretreatment with 6-OHDA reduced total uptake ($\text{Content}_{t=0}$) to 65.29% of control (Control = 100% = $\text{Content}_{t=0}$ of all untreated hearts; n = 121) (Table 3). However, after 60 min efflux, much of the radioactivity was lost so that $\text{Content}_{t=60}$ of 6-OHDA pretreated hearts was only 9.01% of $\text{Content}_{t=60}$ of the untreated group. Exposure of these 6-OHDA pretreated hearts to 56 mM KCl for 30 min commencing at t=60, however, evoked the release of 50.18% of that evoked from untreated hearts (Figs. 21 and 22). Spontaneous efflux was reduced to 18.5% of control.

When 6-OHDA pretreated hearts were perfused throughout (*i.e.*, during loading, passive efflux and KCl treatment) with Krebs solutions containing 10^{-6} M DMI, 10^{-6} M SKF-550, or a combination of both, uptake,

__TABLE 2__. EFFECT OF SKF-550 ON UPTAKE, RETENTION AND RELEASE OF ^{3}H-NA

	CONTENT		RELEASE	
	t = 0	t = 60	Passive	Evoked
CONTROL	497.73±17.54 (121)*	351.17±17.28 (121)	14.16±1.737 (9)	31.39±4.97 (16)
SKF-550	495.12±26.58 (10)	96.58± 6.63 (10)	14.85±1.441 (5)	25.54±2.026 (5)
	n.s.	P<0.01	n.s.	n.s.

* Values as pmol/g, means±S.E.M. (n).

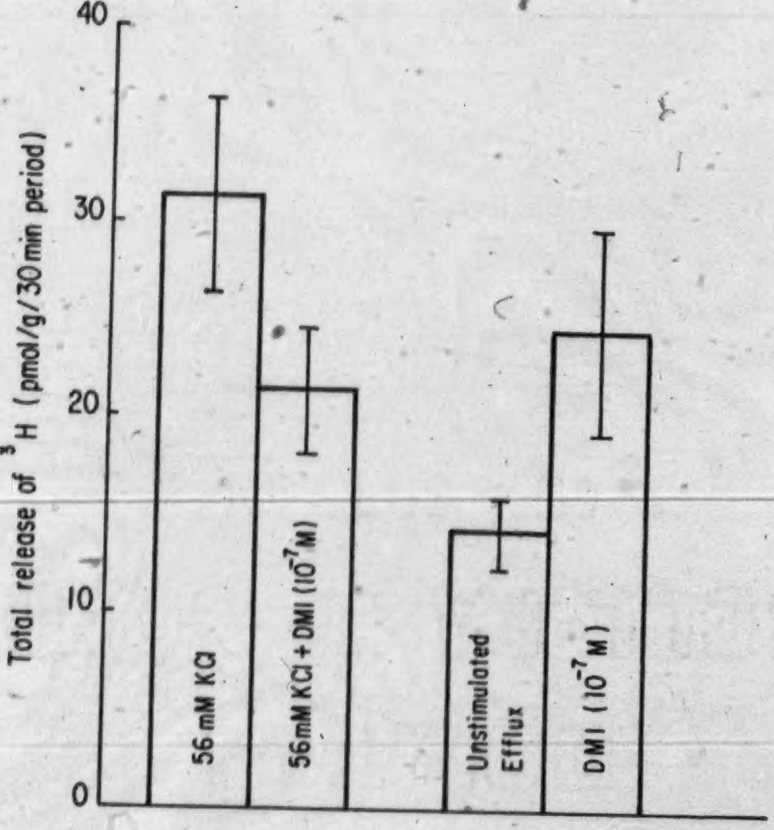

Fig. 20. Effect of DMI on passive and KCl evoked efflux. Means ± S.E.M. For DMI, n = 5; for control, n = 9; for KCl + DMI, n = 6; for KCl, n = 16. The data for this Figure are given in the Appendix, Table 10.

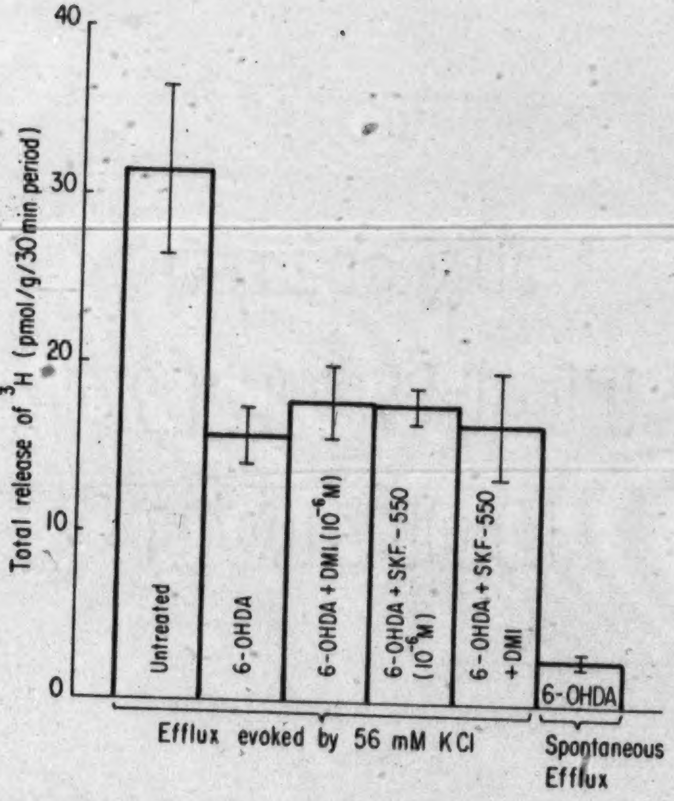

Fig. 21. Effect of uptake inhibitors on KCl efflux following chemical sympathectomy. Means ± S.E.M.; n = 5 except for control, where n=16. The data for this Figure are given in the Appendix, Table 11.

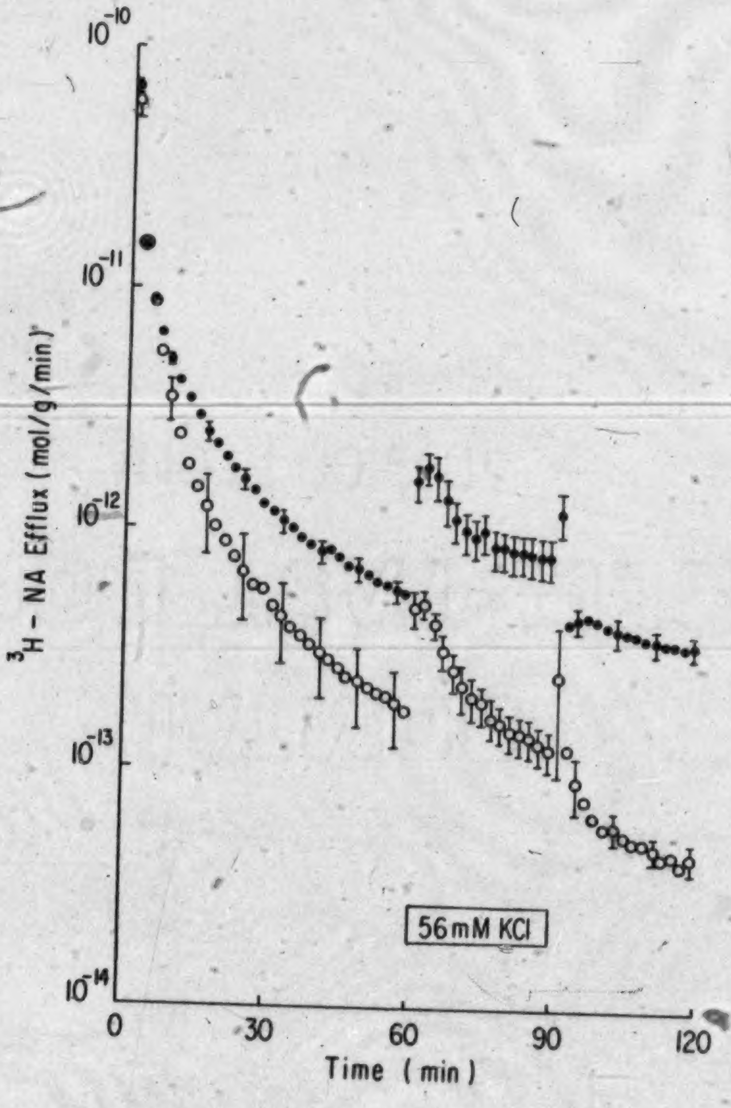

Fig. 22. Effect of chemical sympathectomy on ^{3}H-NA efflux.

Means ± S.E.M. Closed circles = untreated (n = 16)

Open circles = 6-OHDA pretreated (n = 5)

Note - the values from which this graph was plotted are not included in either Table 3 or Table 12.

TABLE 3. EFFECT OF DENERVATION ON UPTAKE, RETENTION AND RELEASE OF ^{3}H-NA

Treatment	CONTENT		RELEASE	
	t = 0*	t = 60*	Spontaneous**	KCl Evoked***
6-OHDA	65.29 ± 13.50(5)	9.01 ± 1.35(5)	18.5 ± 2.94(4)	50.18 ± 5.40(5)
6-OHDA + DMI	66.89 ± 10.14(5)	8.53 ± 1.54(5)	-	56.51 ± 6.94(5)
6-OHDA + SKF-550	77.47 ± 5.34(5)	8.40 ± 0.875(5)	-	56.04 ± 3.48(5)
6-OHDA + SKF-550 + DMI	77.71 ± 3.11(5)	6.19 ± 1.16(5)	-	52.66 ± 10.13(5)
Reserpine	43.06 ± 5.24(5)	4.47 ± 1.09(5)	18.08 ± 2.01(8)	44.80 ± 10.03(5)

* Means ± S.E.M. (n) as % of content of untreated hearts at either t=0 or t=60.

** Means ± S.E.M. (n) as % of spontaneous release from untreated hearts between t=60 and t=90.

*** Means ± S.E.M. (n) as % of release from untreated hearts in the presence of 56 mM KCl Krebs between t = 60 and t = 90.

The data for this Table are given in the Appendix in Table 12.

retention and release values similar to normally perfused denervated hearts were seen (Table 3, Fig. 21).

(i) Content at t=0. There was no significant difference between treatments (treatment A = 6-OHDA, B = 6-OHDA and DMI, C = 6-OHDA and SKF-550, D = 6-OHDA and DMI and SKF-550) and no significant difference between treatment A and treatments B, C and D or between D and treatments B and C.

(ii) Content at t=60. There was a significant ($0.01 < P \leqslant 0.05$) added variance component due to treatments, but no significant difference between treatment A and treatments B, C and D. There was a highly significant difference ($P < 0.01$) between treatment D (both SKF-550 and DMI) and treatments B and C (either DMI or SKF-550).

(iii) Release by 56 mM KCl. There was no significant added variance component due to treatments and no significant difference between treatment A and treatments B, C and D or between treatment D and treatments B and C.

(b) *Reserpine*

It may be seen from Table 3, that pretreatment with reserpine also had a much greater effect on retention at t=60 (3.15% of control) than on content at t=0 (43.06% of control) and that, like chemical sympathectomy with 6-OHDA, its effect on retention was much greater than its effect on KCl induced release (44.8% of control). Spontaneous efflux was reduced to 18.08% of control.

DISCUSSION

Although the release of NA from sympathetically innervated tissues by KCl is a well known phenomenon, there do not appear to have been any attempts to analyse this process in detail. In this present study several questions concerning the mechanism of action of KCl were posed and attempts made to answer them.

(1) Is the release due to KCl or is it an osmotic phenomenon?

(2) Is the release due to an increase in ionic strength or to KCl?

(3) Which of the ionic species (K^+ or Cl^-) is responsible for the release and is this property specific to the active ion or is it a general property of similar monovalent ions?

(4) From what site does the release occur?

(5) By what mechanism(s) does the release occur?

OSMOTIC EFFECT

Since a simple increase in the extracellular osmolarity produced by sucrose did not result in a release of ^{3}H-NA from labelled hearts, it may be concluded that the phenomenon is not due to the increased osmolarity of the high K^+ solution (Fig. 12).

IONIC STRENGTH

If the release is due to an increase in the ionic strength of the high K^+ perfusing solution, then an equivalent increase in ionic strength induced by choline chloride should cause a release. However, there was no significant increase in efflux when such a manouever was performed indicating that release is independent of the ionic strength of the solution. Parenthetically it might be expected that choline would cause a release of transmitter by stimulating nicotinic receptors on noradrenergic nerves in the heart (Farber, 1936; Coon and Rothman, 1940; Burn and Rand, 1960; Daly and Scott, 1961; Brandon and Rand, 1961; Ferry, 1963) as choline is a muscarinic and nicotinic agonist, although much weaker than acetylcholine (Chang and Gaddum, 1933). By increasing the number of experiments, it might be possible to uncover a statistically significant difference between control efflux and efflux in the presence of 56 mM choline chloride and to test the hypothesis that such an increase was due to a nicotinic action, by infusing acetylcholine at a concentration of *ca.* 56 x 10^{-8} M instead of choline chloride. Nash, Cottle and Chang (1971) were able to demonstrate a small release of ^{3}H-NA induced by 10^{-8} M ACh, but owing to the difference between their technique and that used here, it is not possible to make a direct comparison. (In this regard, it is interesting to note that during perfusion with 56 mM choline chloride, the hearts stopped beating, presumably due to a muscarinic inhibition, and then began to beat again at a reduced frequency after 5 to 10 min.)

NATURE OF THE ION RESPONSIBLE FOR RELEASE

The results obtained with choline chloride also suggest that the chloride ion was not responsible for the release induced by KCl. This is not unexpected, as in the event that the release follows a change in the membrane potential of noradrenergic nerves, the alteration induced by Cl^-, if any, would be expected to be an increase in membrane potential, *i.e.*, hyperpolarization, whereas one would assume the stimulus for release to be related to a depolarizing event. The conclusion that the Cl^- ion is unimportant in the release phenomenon is further supported by the observation that 56 mM KCH_3SO_4 produced a small but insignificant increase in NA release relative to that induced by 56 mM KCl. If this small increase is real an explanation may be found in terms of the small hyperpolarization possibly induced by the Cl^- ion. In any event it is clear that the contribution of the Cl^- ion to the NA release is small or negligible and in a different direction to K^+.

The Nernst equation predicts that the membrane potential of excitable cells will be proportional to the log of the external K^+ concentration. If there is some kind of linear relationship between membrane potential and the stimulus for release of transmitter, then one might expect to observe a log-linear relationship between $[K^+]_{out}$ and transmitter release. Experimentally, this was found to apply to ^3H released from hearts by KCl, suggesting that within the limits examined, such a linear relationship applies. This is not to say, however, that each step in the stimulus-secretion system is simply or linearly related to its adjacent step, but rather that the algebraic sum of all such relationships appears to be a linear function of membrane potential. In this interpretation, however, one must bear in mind that there may be a limiting factor at play, which tends to produce such a linear overall relationship. Thus, if the stimulus for release at membrane potentials corresponding to very high external K^+ concentrations is proportionally much greater than that at lower external K^+ levels, the observed release could be limited by the availability of the transmitter store to the release process so that the overall observed relationship is linear. It might be postulated that in a normal sympathetic neurone at rest, the membrane is being constantly bombarded with storage vesicles undergoing random Brownian motion, as it has been shown that random walk alone can account for rates of movement of vesicles quite adequate for rapid exchange between those vesicles near the membrane and those located more centrally in terminal varicosities (Shea and Karnovsky, 1966; Casley-Smith, 1969; Matthews, 1970). At rest very few of these vesicle-membrane collisions might result in exocytotic events, but when the nerve cell becomes depolarized, the properties of the membrane are altered in such a way that the probability of vesicle-membrane interactions resulting in exocytosis is greatly increased. If this probability is a function of membrane potential, then at some point, all vesicles which collide with the membrane will be captured and their contents released. When the membrane potential change exceeds this point, no further increase in release will be observed as the limiting factor is the rate of bombardment which stays constant. This is consistent with the findings of Brown and Gillespie (1957) who found that the amount of NA released per impulse was constant at physiological stimulation frequencies (<10 Hz) but that above frequencies of 50 Hz, the amount of NA released per impulse fell off. It might be argued that on a temporal basis, at least, the depolarization caused by high $[K^+]_{out}$ represents a considerably greater releasing stimulus than stimulation at 50 Hz, so the appearance of a linear overall relationship implies that the membrane potential/releasing stimulus relationship is not linear, or that release is being induced at high $[K^+]_{out}$ by a mechanism which is not subject to the limitations imposed by the availability of storage vesicles.

The graph showing ^{3}H release *vs.* log$[K^+]_{out}$ (Fig. 14) also indicates that the releasing action of KCl does not become apparent until a threshold concentration of about 30-40 mM is reached, which corresponds to a membrane potential of between -30 and -23 mV (assuming the normal resting potential to be -70 mV and the value of $[K^+]_{in}$ to be 100 mM). Such values correspond to the values one might expect for the critical or threshold level at which the action potential is initiated.

These findings appear to be consistent with the postulate that the release of transmitter induced by KCl is due to the initiation of the normal physiological release mechanisms in response to the depolarization of noradrenergic nerve cells produced by the elevated $[K^+]_{out}$. However, such a conjecture is made considerably less appealing by the puzzling finding that 56 mM NaCl also causes a release of ^3H which is quantitatively indistinguishable from that induced by the same concentration of KCl. The effect is again independent of the anion, as 56 mM $NaCH_3SO_4$ evoked the same sized release and may be considered to be due to the Na^+ as the release induced by choline chloride was not significantly different from control. The effect is probably not a mechanical one as, like lithium chloride, sodium chloride did not stop the heart as did 56 mM potassium chloride, nor did it appear to cause the hearts to beat more rapidly or powerfully than usual. As this releasing effect is quite the opposite of that which one might predict on an electrophysiological basis, one can only conclude that the effect is due to some non-neuronal effect. Possible mechanisms for this are:

(a) dilatation of colateral coronary vessels resulting in an increased wash-out of label from heart tissues

(b) release of ^3H-NA from an accessible non-neuronal site, *e.g.*, elastic tissue or collagen by an ion-exchange process.

The first of these possibilities would seem to be unlikely, as one might expect the coronary vessels to be fully dilated as a result of the low pO_2 and generally poor metabolic state of Krebs perfused hearts. In the second case, it would be surprising to find such a close quantitative agreement between the release caused by 56 mM KCl and 56 mM NaCl if they are acting by different mechanisms, which leads one to suspect that at least part of the release induced by KCl could be as a result of a mechanism similar to that responsible for the release induced by Na^+. In addition, the intermediate level of release induced by Li^+ (*i.e.*, not significantly different from control or from KCl), might be due to an action similar to Na^+. In other words, monovalent metallic cations might be able to induce release by means of a similar mechanism, possibly by an ion-exchange process.

SITE OF RELEASE

To attempt to answer the question of whether KCl induces release from nerves or from some other non-neuronal site, the effects of denervation on ^{3}H-NA uptake and KCl evoked release were determined. The finding that pretreatment with 6-OHDA reduced the total tissue content of ^{3}H determined immediately after loading (t=0) to about 65% of control whereas the content after 60 min perfusion was reduced to about 9% of control, suggests that in untreated hearts neuronal uptake of NA represents some 44% of the total content seen at the end of the loading period. The remainder might be expected to represent the ^{3}H-NA in the extracellular space of the heart and that ^{3}H-NA non-specifically bound and that taken into extra-neuronal sites from which efflux is rapid, *i.e.*, complete after 60 min. If the total tissue content of ^{3}H after 60 min efflux can be regarded as an indication of the degree of denervation, then one may infer that the treatment with 6-OHDA has effected a 91% sympathetic denervation. Furthermore, if the release of radioactivity induced by KCl occurs from a sympathetic neuronal site, then the size of the KCl induced release would be expected to be decreased proportionately, *i.e.*, to about 9% of the release evoked by KCl from untreated hearts. In fact, the KCl induced release was only reduced to 50% of control. Similarly, treatment with reserpine, which may be presumed to prevent vesicular sequestration of ^{3}H-NA and thereby reduce the ability of sympathetic nerves to retain and release ^{3}H-NA, reduced the tissue content after 60 min perfusion to only 4% of control, despite which KCl was able to release 45% of control. There are a number of possible explanations for this marked discrepancy between the degree of functional denervation and the ability to release transmitter.

(1) Uptake and KCl evoked release occur primarily into and from a neuronal site which is resistant to both 6-OHDA and reserpine and which is especially sensitive to KCl. However, the close quantitative agreement between release induced from 6-OHDA and reserpine pretreated hearts would seem to argue against this possibility, although such a coincidence cannot be ruled out other than on a conceptual basis.

(2) After treatment with 6-OHDA, uptake occurs mainly into the surviving pre-terminal sympathetic trunks, which do not apparently degenerate (Cottle and Nash, 1974) from which release can be induced more readily than from normal terminals. This could possibly occur if the density of storage vesicles near the nerve membrane is considerably higher than normal or if these vesicles are able to release a considerably greater proportion of their ^{3}H-NA stores than can vesicles in normal peripheral varicosities. Fluorescence studies have shown that these surviving trunks are especially rich in NA (Cottle and Nash, 1974) but there do not appear to have been any electron microscopic studies which indicate whether or not this increased

fluorescence is associated with an increase in the number of vesicles (*cf.* the accumulation of vesicles which occurs proximal to a constriction applied to a nerve trunk - Dahlström and Häggendahl, 1966). Such an explanation does not account for the similar disparity seen after reserpine pretreatment. Again, a separate explanation of the reserpine effect may be postulated. It is possible that vesicles in the so-called "reserpine-resistant" pool of storage vesicles (Hamberger, 1967) are able to take up a larger proportion of label when the other vesicles are blocked with reserpine and that these resistant vesicles subsequently release a much larger proportion of their contents than reserpine-sensitive vesicles normally do. Once again, the criticism that can be leveled at such a set of suggestions is that it would be surprising if two such different mechanisms should result in such quantitatively similar effects.

(3) Perhaps a more acceptable proposal is that normal, untreated hearts which have been labelled with ^{3}H-NA and which have been perfused for 60 min contain at the end of this time a relatively small pool of ^{3}H-NA in an extraneuronal (or non-noradrenergic neuronal) site (<10% of total) and it is from this site that much of the ^{3}H-NA comes when the hearts are treated with KCl. Such a site would not be subject to depletion or destruction by 6-OHDA or reserpine and consequently, such treatments would abolish the concurrent release from noradrenergic nerves leaving only the release from this other site. Possible candidates for this site are:

(a) non-noradrenergic nerves, *e.g.*, cholinergic, dopaminergic, tryptaminergic, purinergic

(b) a non-specific binding site, *e.g.*, NA adsorbed onto collagen or elastic tissue

(c) non-neuronal cells, *e.g.*, cardiac muscle cells, vascular smooth muscle cells, epithelial cells.

Of the non-noradrenergic neuronal candidates, only cholinergic nerves are known to exist in the heart and there is no evidence to suggest that these nerves either possess a mechanism by which NA may be taken up and retained or are capable of releasing a transmitter other than ACh or very close structural analogues of ACh. Of the other two possibilities, *viz.*, retention in a non-specific, possibly surface, site and an intracellular uptake and retention by non-neuronal cells, neither is particularly more likely than the other. Perhaps the log-linear relationship between release and K^+ concentration argues in favour of release from an intracellular site and particularly from a cell type which is electrically sensitive, *e.g.*, muscle cells. However, the finding that release can be induced by NaCl argues against this and possibly suggests an ion-exchange type of mechanism from a surface site. There would seem to be no reason why such an ion-exchange mechanism or even a desorption phenomenon should not be dependent upon the log of the cation concentration. If the concentration *vs.* release relationship for NaCl is similar to the pattern found with KCl, then one may perhaps conclude that the release cannot be as a result of depolarization of excitable cells.

In order to provide evidence which might distinguish between these two possible non-neuronal uptake-retention-release sites, and to exclude the possibility of involvement of noradrenergic nerves, drugs known to interfere with the two clearly defined uptake processes were used, namely, SKF-550 (inhibitor of extraneuronal uptake) and DMI (inhibitor of neuronal uptake). SKF-550 markedly reduced the ability of otherwise untreated hearts to retain ^{3}H-NA (content$_{t=60}$ reduced to 27% of control) implying that normally a large proportion of the total tissue stores of ^{3}H-NA are either retained for longer than 60 min in a non-neuronal site, or that at the end of a 60 min efflux period, much of the total ^{3}H-NA in neuronal stores has passed through an extraneuronal storage site. This latter possibility is perhaps supported by the fact that there was no difference between the total tissue contents at t=0 of hearts loaded in the presence and absence of SKF-550. The subsequent finding that SKF-550 had no effect on either the passive efflux between t=60 and t=90 or on the KCl evoked efflux is yet a third instance of a discrepancy between tissue content and release. This may be interpreted as indicating that the uptake process into the site from which much of the KCl induced release occurs is not inhibited by SKF-550. In other words, it is not a "classical" extraneuronal uptake site.

A similar picture was obtained when uptake, retention and KCl induced release were determined in 6-OHDA pretreated hearts which were loaded and perfused with SKF-550 present in the Krebs solution. There was no difference, however, between the contents at t=60 between SKF-550 and normally perfused denervated hearts. This may imply that at the dose used (10^{-6} M) SKF-550 does, in fact, partly inhibit neuronal uptake. However, if the postulate made above (that the neuronal store is partly maintained by relocation of ^{3}H-NA which has been through an extraneuronal site) is correct, then when the neuronal site is destroyed, such a redistribution of ^{3}H-NA could occur in neither the

SKF-550-treated nor the untreated group and no difference would therefore be expected.

As with the normal hearts, 6-OHDA denervated hearts treated with SKF-550 were still able to respond with a release of ^{3}H-NA when exposed to KCl, further demonstrating the independence of the release site from a prior uptake process governed by classical extraneuronal uptake mechanisms.

After pretreatment with 6-OHDA, DMI, like SKF-550, had no effect on uptake, retention or KCl evoked release, further emphasizing the relative unimportance of a neuronal storage site for release in response to KCl. However, when DMI and SKF-550 were combined, there was a significant difference in the retention at t=60 between hearts treated with either DMI or SKF-550, which suggests that in fact in 6-OHDA pretreated hearts there are still small components of both neuronal and extraneuronal retention at t=60, which when added together bring the difference within the range of significance. It should be noted, though, that combined SKF-550 and DMI had no significantly different effect on release than either alone or no treatment, as would be expected if release after 6-OHDA is from a non-neuronal site entry into which does not obey the kinetics of classical extraneuronal or neuronal uptake.

If it is assumed that SKF-550 inhibits extraneuronal uptake in the same way as does phenoxybenzamine, another β-haloalkylamine, then one would expect that uptake into smooth muscle cells, cardiac muscle cells and epithelial cells would be inhibited, but not that accumulation in or on collagen and elastic tissue (Eisenfeld, Axelrod and Krokoff, 1967; Eisenfeld, Landsberg and Axelrod, 1967; Avakian and Gillespie, 1968; Lightman and Iversen, 1969; Gillespie, Hamilton and Hosie, 1970; Draskoczy and Trendelenburg, 1970; Iversen, Salt and

Wilson, 1972). Perhaps, then, the site from which release can be evoked by KCl is an extracellular site, for example, collagen and/or elastic tissue. An argument against this is that on the basis of fluorescence microscopy studies, Avakian and Gillespie (1968) found uptake or binding of NA to such presumably extracellular sites to be very rapidly exhausted during washout (10 min), whereas the intracellular sites maintained a considerable degree of fluorescence for long periods of time, although with the concentrations of NA used in their study (6×10^{-4} M), fluorescence was scarcely different from pretreated levels after only 30 min. One must bear in mind, however, that considerable amounts of NA could still be bound in these respective sites over and above their normal levels and not produce a detectable fluorescence, in addition to which, extraneuronal binding of labelled NA by means of an exchange process with endogenous unlabelled NA bound to the same site would produce an increase in the specific activity of such bound NA without an overall change in the total NA content of such a site. In this case, no alteration in the "background" fluorescence would be observed, although the site would be loaded with tracer which could be displaced and detected in efflux experiments. Another possibility, and one with considerable appeal, is that the ^{3}H species which is detected during KCl treatment is not ^{3}H-NA, but a metabolite (which would not show up on fluorescence microscopy) and which is only slowly washed out and binding or uptake of which is not inhibited by the classical extraneuronal uptake inhibitor SKF-550. To test this possibility, it should be possible to perform an analysis of the metabolites in the effluent during KCl treatment, using untreated hearts, 6-OHDA pretreated hearts both in the presence and absence of SKF-550.

One further point should, perhaps, be made regarding the effect of denervation with 6-OHDA on the distribution of ^{3}H-NA. In the normal heart, there is a competition between the different uptake sites (*i.e.*, neuronal and non-neuronal) for the infused ^{3}H-NA. After 6-OHDA, however, the neuronal component is effectively removed, which means that the effective concentration of ^{3}H-NA to which the other sites are exposed is higher. It is highly likely, therefore, that following 6-OHDA the proportion of the infused ^{3}H-NA taken into non-neuronal sites is somewhat (or even considerably) higher than in untreated hearts, so that the amount of ^{3}H-NA released from these sites may be equivalently more than that released from these same sites in untreated hearts (*i.e.*, those with intact adrenergic systems). Although such a phenomenon will distort the estimates of the relative sizes of these neuronal and non-neuronal compartments, it does not explain why there is such a discrepancy between retention capacity and releasing capacity in 6-OHDA pretreated hearts.

5. *MECHANISM(S) OF RELEASE*

One way in which KCl could cause a net efflux of ^{3}H-NA is by the inhibition of the neuronal membrane re-uptake system so that a portion of the transmitter which leaks out of nerves spontaneously but which is normally recaptured appears as a net increase in efflux when this re-uptake is inhibited. In order to study the effect of KCl on uptake quantitatively, it was necessary to obtain a correction term for the tissue extracellular space penetrated by ^{3}H-NA during the uptake period as this approach was felt to be more likely to give reliable estimates of initial uptake rates than the washout procedure (Iversen, 1963). ^{14}C-D-Mannitol, a readily available and commonly used extracellular space marker, was chosen for this purpose.

In the presence of 112 mM KCl, there was no significant increase in the extracellular space (V_t) which suggests that the cell shrinkage one might expect at high [KCl] was minimal. In the presence of 0.5 µM NA, however, there were highly significant increases in the value of V_t at all times studied except after 1 min. As these values approached or even exceeded 100%, it is clear that some kind of concentration of the marker had occurred and it was decided to use the value of 40% for a 5 min uptake exposure (*cf.* Nash, Gillespie and Robertson, 1974). The reasons for this concentration phenomenon in the presence of NA are uncertain and, although not directly pertinent to this present study, deserve brief discussion and further experimental work.

Possible mechanisms for this apparent increase in extracellular space would be:-

(a) Alteration in membrane permeability to mannitol.

As the total tissue water can never reach 100%, it is inconceivable that the high values obtained resulted solely from the NA rendering cell membranes freely permeable to mannitol.

(b) Stimulation of an uptake process.

An alternative to (a) would be that the NA stimulates a normally inactive uptake process which is either selective for D-hexoses like mannitol or which is non-selective. Another possibility is that the action of NA is to greatly alter the specificity of an existing and active uptake mechanism. From a phylogenetic standpoint, it would seem most unlikely that such a bizarre uptake process should occur in a mammalian or any zoological system, although one may not condemn such a proposal without experimental evidence, none of which is available.

(c) Stimulation of a binding process.

A third and perhaps more plausible explanation is that NA in some way activates a binding or sequestration process which, although possibly occurring in a minute fraction of the heart's mass, is sufficient to produce an overall apparent uptake such as that seen. Stimulation of such a binding or sequestration process may be envisaged as one of the following:-

(i) a pharmacological action of NA in diverting the fluid flow through vascular beds which are not normally open to free perfusion and which contain sites which are physico-chemically or biochemically capable of binding mannitol, *e.g.*, adsorption onto collagen.

(ii) a direct action of NA on some site which is normally exposed to perfusion fluid which results in a change in the properties of that site in such a way that mannitol is bound, *e.g.*, alteration in cell membrane structure so that mannitol becomes adsorbed or dissolves more readily in cell membranes.

(iii) an activation by NA of an enzyme system capable of metabolizing mannitol resulting in the liberation of a ^{14}C-labelled degradation product which may be bound or chemically incorporated by cells.

Of these possibilities, the second seems most attractive as it does not involve the postulation of an unusual enzyme or a drastic alteration in the hydrodynamics of the perfused heart and although this latter is a distinct possibility it would seem improbable that the uptake/binding sites it requires are accessible only when such an hydrodynamic change is effected. This is also the most aesthetically attractive and might be amenable to investigation by the examination of the binding properties of mannitol to various subcellular fractions under a variety of pharmacological conditions. It is also possible that the binding site is intracellular and that NA alters the membrane permeability in such a way that mannitol enters cells by a passive diffusion process and is bound, thereby maintaining a concentration gradient into the cells.

EFFECT OF KCl ON ^{3}H-NA UPTAKE

The finding that removal of K^+ alters the maximum uptake velocity (V_{max}) without altering the affinity for the postulated uptake carrier (Fig. 17) supports the findings of White and Paton (1972), who found a similar effect of reduced [K^+] on the uptake of NA into synaptosomes prepared from whole rat brains. In this present study, the rate of NA uptake at elevated levels of K^+, was reduced, although not totally inhibited by any of the concentrations used (up to 230 mM) and in fact any alteration of [K^+] away from the normal 6 mM impaired NA uptake. An exception to this was apparent when the concentration of NA was 0.05 μM, the highest used in this study. In this case, the optimum [K^+] concentrations for uptake appeared to be about 30 mM. This difference could be because the kinetics of NA uptake at this concentration of NA are different from those at lower concentrations, which could arise if at the higher concentration, an extraneuronal component (Uptake$_2$) contributed to the overall uptake and was less sensitive to K^+ than the uptake processes at lower concentrations of NA. Alternatively, there could be a distinct difference between neuronal uptakes above and below 0.05 μM NA. Iversen (1963), using NA concentrations up to 2.4 μM obtained a value of 0.27 μM for the K_m of ℓ-NA uptake by rat hearts, whilst White and Paton (1972) obtained a value of 0.8 μM. In this present study the K_m was found to be 0.094 μM indicating an affinity for the NA carrier nearly 3 times higher than that found by Iversen (1963) and nearly 9 times that of White and Paton (1972). The value for K_m determined by these latter workers is 3 times that found by Iversen (1963) and this could be because they used synaptosomes prepared from whole rat brains which would include pinched off nerve endings from a variety of neuronal types. If any of these neuronal species possess uptake processes which will transport NA at reasonably high rates, albeit with low affinities, the overall value of K_m will be artificially elevated, as the overall value of V_{max} will be raised. However, in this present study, a system very similar to that used by Iversen (1963) was used, except that

(1) he used a constant pressure perfusion system, rather than constant flow,

(2) he allowed uptake to occur for up to 60 min and extrapolated back to time zero to obtain initial uptake rates,

(3) he employed higher concentrations of NA,

(4) he used a 2 min washout period to clear the extracellular space of ^{3}H-NA.

It is possible that the extrapolation technique and the use of a constant pressure system resulted in overestimates of the initial uptake velocity, which would result in high values for K_m (*i.e.*, a low affinity). However, it is also conceivable that the low value for K_m found in this study arose because at the very low concentrations of NA employed, another high affinity uptake process is at work. This would seem unlikely and it is more probable that the 3-fold difference is a product of the differences between the techniques.

Thus, as inhibition of re-uptake by high concentrations of K^+ is a possible contributory factor to the release phenomenon, it is important to know what proportion of the spontaneously released ^{3}H-NA is normally recaptured by the re-uptake process. Treatment with 10^{-6} M DMI for 30 min yielded a release of ^{3}H as large as that released by 56 mM KCl, which suggests that quite a large proportion of the spontaneously released NA is normally re-captured. However, as 56 mM KCl did not produce more than about 50% inhibition of uptake at any of the concentrations of NA studied, (except at 5 x 10^{-8} M, which is possibly a higher concentration than the nerve cells are exposed to during normal efflux) some of the release evoked by 56 mM KCl must result from an increase of the gross efflux from storage sites. If this is the case, then treatment with both 56 mM KCl and 10^{-6} M DMI should have a partly additive action, as a proportion of the NA released by KCl *per se* will be normally subject to re-uptake. The finding that there was no additive effect, however, mitigates against this concept. While the local anaesthetic properties of DMI could provide some explanation of the absence of an additive effect, Greeff and Wagner (1969) report the local anaesthetic potency of DMI to be only about 0.4 times that of cocaine, so that a complete nerve trunk block might be expected to be produced by DMI at a concentration of about 6 x 10^{-3} M, which is several orders of magnitude greater than that used (10^{-6} M), making local anaesthesia an improbable explanation. However, Hrdina and Ling (1970) presented evidence that at low concentrations (above 10^{-7} M) DMI interferes with stimulus-contraction coupling by altering in some way the availability of Ca^{++} to the contractile mechanism and in another report (Hrdina and Garattini, 1967) it was demonstrated that DMI produced a relaxation of arterial smooth muscle previously contracted by depolarizing concentrations of K^+, an effect that was abolished by raising the Ca^{++} concentration. Thus, although 10^{-6} M DMI can cause an apparent release on its own by inhibiting re-uptake, in the presence of DMI, K^+ may be unable to cause any release itself as Ca^{++} availability is impaired. This hypothesis is consistent with the finding of Nash *et al.* (1972) that Ca^{++} ions were essential for the NA releasing action of K^+.

Ca++ has also been shown to be essential for release of catecholamines from adrenal medulla (Douglas and Rubin, 1961) and for the release of NA from rabbit heart, rabbit ileum and cat spleen on nerve stimulation (Hukovic and Muscholl, 1962; Boullin, 1965; Kirpekar and Misu, 1967).

One may conclude, then, that although the release induced by KCl is not primarily a result of inhibition of re-uptake of spontaneously released NA, the effect is enhanced by a partial inhibition of re-uptake of NA released by the KCl.

OTHER MECHANISMS

The denervation studies described above showed that only some 50 - 60% of the release induced by KCl comes from neuronal sources and it would seem probable that most of this release is evoked by means of depolarization of sympathetic nerves.

As has been discussed above, the remainder of the released ^3H is coming from an extraneuronal site, possibly an extracellular binding site. It would seem that the most probable mechanism by which such a release could be induced by monovalent cations would be an ion-exchange process. This would require that the bound tritiated species be also cationic. As the major metabolites of NA are anionic, it is highly likely that the displaced label, seen when hearts are perfused with high KCl solution, is attached to the parent compound, NA. The O-methylated derivate of NA (normetanephrine) is also cationic at physiological pH and so it is equally possible that this species is released along with ^3H-NA from the postulated extracellular binding site.

It may be argued that the equation describing such an ion-exchange mechanism would show a linear relationship between concentration and displacement, unlike that shown in Fig. 14, where the release of ^{3}H-NA is approximately proportional to the logarithm of the K^+ concentration. Such a logarithmic relationship could, however, appear if the process can be described by the type of equation which describes the Langmuir adsorption isotherm, in which a plot of adsorption against concentration is a rectangular hyperbola. This can be converted to sigmoid curve by plotting adsorption against log . concentration and this type of curve is approximately linear in its middle part (*c.f.*, Log . dose-response curves, which show a striking parallel with the Langmuir isotherm) (Adam, 1941).

SUMMARY OF EVIDENCE

The experiments that have been performed clearly cast doubt on the belief that KCl causes release of radioactivity solely from a noradrenergic neuronal pool and further doubts upon the mechanism being simply one of membrane depolarization. In order to facilitate the assessment of the possible alternatives these are presented below, along with the evidence which supports them.

1. *RELEASE OCCURS FROM A NEURONAL SITE*

(a) Stimulation of release was shown to be independent of the anion, in keeping with the predictions of the Nernst equation, assuming that the Cl^- ion contributes little to the maintenance of the resting potential.

(b) The amount of radioactivity released was approximately proportional to log $[K^+]_{out}$ (above a threshold of *ca.* 35 mM), which again agrees with the predictions of the Nernst equation.

(c) Perhaps the best evidence for the neuronal theory comes not from the experiments presented here, but from the work of the many others who have clearly shown that

(i) high $[K^+]_{out}$ causes depolarization and firing in nerves

(ii) transmitter release from both cholinergic and noradrenergic nerves is closely related to the nerve membrane potential.

2. *RELEASE OCCURS FROM A NON-NEURONAL SITE*

(a) Following 6-OHDA sympathectomy, there was a marked discrepancy between tissue content and KCl evoked release, *i.e.*, content was reduced to 9% but release to only 50% of normal.

(b) When 6-OHDA pretreated hearts were perfused throughout with DMI (10^{-6} M) to inhibit uptake into any residual nerves, KCl evoked release was not further impaired.

(c) Like 6-OHDA, reserpine had disparate effects upon ^{3}H-NA content and release in response to KCl; content was reduced to 4% but release to only 45% of normal.

On the basis of this evidence, it is not possible to conclude that KCl evokes no release from neuronal sites - on the contrary, the denervation experiments suggest that approximately half of the observed release from normal hearts is from noradrenergic neurones and it is probable that most of this results from the depolarizing action of K^+. However, in view of the demonstration of uptake inhibition in the presence of elevated K^+, it appears that a proportion of the apparent release from noradrenergic nerves is due to the reduced re-uptake of spontaneously released ^{3}H-NA. This spontaneous release (leakage) need not be only from nerves, as it could well be from non-neuronal sites, into which the K^+ inhibited uptake may also have been directed.

We may thus conclude that some 50% of the KCl evoked ^{3}H-NA release emanates from a non-neuronal site. That this site is not filled via the Uptake$_2$ mechanism was shown by the experiments using 6-OHDA pre-treatment together with SKF-550. The mechanism(s) of release from this non-neuronal site are not easily assessed, but some possibilities are:

(a) release from a non-neuronal, intracellular site (*e.g.*, muscle cells) by depolarization

(b) desorption from extracellular binding sites by the elevated ionic strength

(c) washout from the poorly perfused parts of the coronary capillary bed as a result of altered haemodynamics

(d) ionic-exchange between ^{3}H-NA (cationic at pH 7.4) or cationic metabolites and mono-valent metallic cations at some extra-cellular binding sites.

Of all these various possibilities, the one which is most consistent with the experimental findings, including the paradoxical releasing action of Na^+, is the last and so it should be considered as a hypothesis and subjected to further study.

In conclusion it may be said that the KCl induced release of ^3H from perfused rat hearts in which the sympathetic transmitter stores have been labelled with ^3H-NA is independent of the Cl^- ion and is not due to either the increased osmolarity or ionic strength of the solution and the release is related to $\log[K^+]$. Monovalent cations other than K^+ also cause release, their order of potency being $K^+ > Na^+ > Li^+$.

As K^+ is a partial inhibitor of NA uptake, KCl-induced release is mediated partly through a release *per se* and partly by an inhibition of the re-uptake process(es).

The denervation studies indicate that the release of ^3H-NA from neuronal stores accounts for only some 50-60% of the total release induced by 56 mM KCl, the entry of ^3H-NA into the extraneuronal site(s) from which the remainder of the ^3H release occurs not being by means of the $Uptake_2$ mechanism. Instead, it is postulated that the extraneuronal site comprises ^3H-NA or cationic metabolites (^3H-normetanephrine) bound to extracellular components of the heart from which release may be induced by the ion-exchange mechanism.

In view of these findings it is suggested that the results of past and future experiments in which sympathetic nervous activity is presumed to be simulated by the use of KCl should be viewed with caution.

SUGGESTIONS FOR FURTHER STUDIES

(a) *Effect of NA on Determinations of Extracellular Space*

The unexpected finding that NA (5×10^{-7} M) caused an apparent uptake of ^{14}C-mannitol is worthy of further study. The following questions would be appropriate:-

(i) Is the effect seen only with mannitol or are other markers, *e.g.*, sorbitol, sucrose and inulin similarly affected and if so, what relation exists between the effect and molecular size and structure of the marker?

(ii) Is the phenomenon dependent upon intact cells or is it exhibited by fractions prepared from homogenates of whole tissue?

(b) *Nature of the ^{3}H-labelled Compounds Released by K^+, Na^+, and Li^+.*

Since Haeusler, *et al.*, (1968) were unable to demonstrate any release of endogenous NA from perfused cat hearts by LiCl, and since Na^+ would not be expected to cause a release of transmitter from nerves by means of a depolarization, the apparent releasing actions of these two cations deserves further study. As a result of the study presented above, it has been postulated that K^+, Na^+ and Li^+ may cause a release of ^{3}H-NA and/or cationic metabolites by means of an ion-exchange mechanism. Consequently, an analysis of the metabolites of ^{3}H-NA present in the perfusate from normal and denervated hearts, during perfusion with both normal Krebs and Krebs containing excess KCl, NaCl or LiCl may shed light on this problem. It might also be profitable to determine whether these cations are able to cause a release of these metabolites from sub-cellular fractions to which they might be bound.

(c) *Comparison of Nerve Stimulation and KCl*

Although the preparation of an isolated, perfused rat heart with an intact sympathetic innervation is technically feasible, it would be difficult. However, it is possible to stimulate nerves selectively by field stimulation, so that it may be possible to compare release of ^{3}H-NA and ^{3}H-labelled metabolites induced from perfused rat hearts by KCl and field stimulation. However, as there are many other sympathetically innervated preparations available (*e.g.*, isolated, perfused rabbit heart, hypogastric nerve - vas deferens, innervated intestinal smooth muscle) it may be more convenient to conduct such comparisons using one of these. The questions which might be posed are:

(i) Are the ionic requirements for release (particularly Ca^{++}) the same for electrical stimulation, KCl, NaCl and LiCl?

(ii) Are the metabolite profiles during release the same?

(iii) What are the ionic requirements and metabolic profiles when release is induced from sympathectomized tissues either electrically or by cations?

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APPENDIX

PROGRAM 1. APL/360 PROGRAM TO PROCESS EFFLUX DATA

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     ∇ EFFLUX2;A;B;BG;C1;C2;F;TT;T2;D;CPM;R;Q;E;T;XX;HC;I;J;RC;CC;C1C;M;HM;C;CR;CHANNELS;K;RS
[1]    DIGITS 4
[2]    'ENTER THE STANDARD DPM AND COUNTING TIME:'
[3]    D←⎕
[4]    'ENTER BACKGROUND COUNTS:'
[5]    BG←+/BG÷⍴,BG←⎕
[6]    'ENTER THE STANDARD COUNTS:'
[7]    CPM←(CPM←⎕)-BG
[8]    'ENTER THE CHANNELS RATIOS IN THE SAME ORDER:'
[9]    R←⎕
[10]   CHANNELS←⍉(2,⍴R)⍴Q←R,E←(CPM÷D[1])×100÷D[2]
[11]   T←(⎕×⍴⍴⍞←'ENTER FLOW RATE, COUNTING TIME AND TIME BETWEEN SAMPLES'),XX←HC←I←J←RC←CC←C1C←⍳0
[12]   (2 1)⍴' '
[13]  LABEL2:(2 1)⍴' '
[14]   →(0=+/M←⎕)/HP1,⍴⍞←'ENTER COUNTS IN HEART, CHANNELS RATIO AND WEIGHT OF HEART'
[15]   'ENTER DILUTION FACTOR FOR HEART CONTENT (ENTER ''1'' IF NO CORRECTION DESIRED)'
[16]   HC←⎕
[17]   HM←500×(M[1]÷T[2])÷(1÷(M[2],M[2]) INTERPOLATE CHANNELS)
[18]   C←(C←⌽(⌽⎕),⌽⎕×⍴⍴⍞←'ENTER COUNTS IN SAMPLES, HIGH COUNTS FIRST:')-BG
[19]   →((⍴C)=⍴CR←⌽(⌽⎕),⌽⎕)/HP2,⍴⍞←'ENTER IN SAME ORDER THE CHANNELS RATIOS OR ''CR''IF SAME AS LAST HEART'
[20]   →HP3,C←100×(C÷(+/CR INTERPOLATE CHANNELS)÷⍴CR)
[21]  HP2:C←100×(C÷CR INTERPOLATE CHANNELS)
[22]  HP3:CC←CC,C←⌽((T[1]×T[3])÷M[3])×C÷T[2]
[23]   (2 1)⍴' '
[24]   '      TIMES   DPM  CUMULATE DPM    ∘/∘'
[25]   '      (MIN)   PER G     PER G     EFFLUX'
[26]   C2←(C÷C1←(HC×(HM÷5)×M[3])+C+.×(⍳⍴C)∘.≤⍳⍴C)×33.333333
[27]   TT←(0.5+(T2←⌽T[3]×⍳⍴C),C,C1
[28]   ⌽[1]⍉(4,⍴C)⍴TT,C2
[29]   (2 1)⍴' '
[30]   'CORRECTED DPM OF HEART ARE: ';(0.5+HC×(HM÷5)
[31]   'DO YOU WANT AN INDIVIDUAL PLOT?'
[32]   →('NO'∊⍞)/LABEL5
[33]  LABEL4: 40 80 PLOT⍉(2,⍴C)⍴T2,10⍟C2
[34]  LABEL5:→((⍴XX)>⍴J)/LABEL1
[35]   →LABEL2,(RC←RC,C÷T[3]),C1C←C1C,C2
[36]  HP1:(2 1)⍴' '
[37]   'DO YOU DESIRE A PLOT OF AVERAGE ∘/∘?'
[38]   →LABEL1×⍳2=⍴K←⍞
[39]   (2 1)⍴' '
[40]   '   TIMES         AVE∘/∘'
[41]   ⌽[1]⍉(2,((⍴XX)÷2))⍴XX←T2,4 AND C2←(+/[1](RS,⍴C)⍴C1C)÷RS←(⍴C1C)÷⍴C
[42]   →LABEL4
[43]  LABEL1:(3 1)⍴' '
[44]   'DO YOU WISH TO PROCESS ANOTHER EXPERIMENT?'
[45]   →1×⍳3=⍴F←⍞
[46]   (5 1)⍴' '
[47]   '
[48]   '                    ∘∘∘∘  ∘   ∘  ∘∘'
[49]   '                    ∘     ∘∘  ∘  ∘ ∘'
[50]   '                    ∘∘∘   ∘ ∘ ∘  ∘  ∘'
[51]   '                    ∘     ∘  ∘∘  ∘ ∘'
                            ∘∘∘∘  ∘   ∘  ∘∘'
     ∇
```

PROGRAM 1a. APL/360 INTERPOLATION SUB-ROUTINE

```
    ∇ R←A INTERPOLATE B;I;X;Y;Z
[1]   SET←0
[2]   I←ρR←⍳1-ρρB←⍉(ρ⍉B)ρ(,⍉B)[Z,(ρB)[1]+Z←⍋B[;1]]
[3]   →((I←1+I)>ρA)/0
[4]   →((A[I]>⌈/B[;1])∨A[I]<⌊/B[;1])/L1
[5]   →(∨/A[I]=B[;1])/L2
[6]   →3,R←R,(A[I]=B[;1])/B[;2]
[7]  L2:→3,R←R,(A[I]×Y[1])+(Y←B[X,1+X;1] LSQPLOT B[X,1+X←+/A[I]>B[;1];2])[2]
[8]  L1:→(SET= 1 0 ¯1)/3,L3,L4
[9]  L4:→ρR←⍳1-ρρR←'ONE OF THE VALUES IS OUT OF RANGE'
[10] L3:R←R,(A[I]>⌈/B[;1])/(A[I]×Y[1])+(Y←(B[X;1]) LSQPLOT B[(X←(ρB)[1],¯1+(ρB)[1]);2])[2]
[11]  →3,R←R,(A[I]<⌊/B[;1])/(A[I]×Y[1])+(Y←B[1 2 ;1] LSQPLOT B[1 2 ;2])[2]
    ∇
```

PROGRAM 1b. APL/360 PLOT SUB-ROUTINE

```
    ∇ A PLOT B;C;D;F;G;N;I;J;L;T;Y;NZ;NB;VT;PT;ST;ISV;U
[1]   PC←'⊖⍟⋆∇Δ⎕'
[2]   NS←0
[3]   SX← 1 2 5
[4]   SM← 5 10
[5]   →((0=×/(2ρA),ρB), 3 2 1 <ρρB)/0,PL7,PL1,PL2
[6]   →PL2,ρB←⍉(2,D)ρ(⍳D←ρ,B),B
[7]  PL1:B←B[1;;]
[8]  PL2:Y←1+⍳(ρB)[2]-1
[9]   C←((⌈/⌈/B[;Y])-⌊/⌊/B[;Y]),(⌈/B[;1])-⌊/B[;1]
[10]  F←|(2ρA)÷C+(C=0)×B[1; 2 1]+B[1; 2 1]=0
[11]  F←(SX[+/((⌊0.0001+F×10⋆-G)∘.≥SX])×10⋆G←⌊10⍟F
[12]  G←(SM÷F)×⌊(((⌊/⌊/B[;Y]),⌊/B[;1])×F÷SM←16|SM[ 1 4
[13]  B[;1]←⌊0.5+F[2]×B[;1]-G[2]
[14]  B[;Y]←⌊0.5+F[1]×B[;Y]-G[1]
[15]  N←SM×⌈(((⌈/⌈/B[;Y]),⌈/B[;1])÷SM
[16]  NB←G[1]+(SM[1]÷F[1])×0,⍳N[1]÷SM[1]
[17]  NZ←G[2]+(SM[2]÷F[2])×0,⍳N[2]÷SM[2]
[18]  0ρST←6ρ~ISV←1=U←9
[19] PL3:VT←∨/0>NB←NB×10⋆U-ST[6-ISV]←I←1+⌈/⌊10⍟|(NB≠0)/NB
[20]  PT←⌊1+10|PT-1|PT←1E¯5+(|NB)∘.÷10⋆¯1+⌽⍳U
[21]  L←U+1-(⌽((C←ρNB)ρ1)∧.=PT)⍳0
[22]  →((U>T←VT+⌈/I,(L+L≠I),(I≤0)×2+L-I),ST[2-ISV]←ST[2-ISV]∨L≥U-VT+L>I)/ 3 2 +⌶26
[23]  →(¯1+⌶26),ρST[4-ISV]←I-1
[24]  →PL3,ρNB←SM[1+~ISV]×¯1+⍳C
[25]  PT←(-VT+0⌈1-I)⌽PT
[26]  PT←(,PT)×J←,⍉(⌽ρPT)ρ(,⍉(1≠PT)∨.∧(⍳U)∘.≤⍳VT[I-1]),(C×U+1-I←VT+I⌈I≤0)ρ1
[27]  →(~VT)/2+⌶26
[28]  PT[(U-+/(C,U)ρJ)+U×¯1+⍳C]←11×NB<0
[29]  PT←(~(⍳U+J)∊(I+J),⍳¯1+J←U-T)\(1 0 +C,U)ρPT,Uρ0
[30]  PT[⍳C;I+J]←12
[31]  PT←' 0123456789¯.'[1+PT[;⍳U-1]]
[32]  →(~ISV)/PL6
[33]  L←1,N[2]ρ0×C←N[1]
[34] PL4:L←(L×NS×C≠0)⌈1,N[2]ρ0
[35]  L[1+(D≠0)/B[;1]]←(D≠0)/D←(C=B[;Y])⌈.×Y
[36]  →(C≠0)/PL5
[37]  L←L⌈0=(SM[2]÷2)|0,⍳N[2]
[38] PL5:PT[((ρPT)[1],1+C÷SM[1])[1+0=SM[1]|C];],(' |',(ρY)ρPC)[1+L]
[39]  →(0≤C←C-1)/PL4
[40]  →(U=U←SM[2]-~ISV←~ρρNB←,NZ)/PL3
[41] PL6:(SM[2]-9)⌽(,(0 0 ,(U-1)ρ1)\PT),' '
[42]  →(ST[1 3 2 4],1)/ 1 3 5 7 10 +⌶26
[43]  'ORIGIN AND SCALE FACTOR FOR ORDINATE: ';G[1],÷F[1]
[44]  →(0=ST[3])/2+⌶26
[45]  'SCALE FACTOR FOR ORDINATE: ';10⋆ST[5]-1
[46]  →(0=ST[2])/2+⌶26
[47]  'ORIGIN AND SCALE FACTOR FOR ABSCISSA: ';G[2],÷F[2]
[48]  →(0=ST[4])/0
[49]  'SCALE FACTOR FOR ABSCISSA: ';10⋆ST[6]-1
[50]  →0
[51] PL7:'THE RIGHT ARGUMENT OF  PLOT  MUST HAVE RANK ≤ 3.'
    ∇
```

The program entitled "HEFFLUX2" (Program 1) is an interactive program written in APL/360 and takes manual (keyboard) input of raw data from efflux experiments and converts them to

(a) efflux, in dpm/g/min,

(b) total activity remaining in the heart, in dpm/g,

(c) efflux coefficient, expressed as %.

The program begins by requesting input of the radioactivity (in dpm) contained in each of the quenched standard vials and the counting time used. This program was designed for use on a scintillation counter in which samples were counted for a preset length of time. If the Beckman LS-230 is used, enter "1". The user then enters the background counts followed by the counts obtained from the standard vials, these data being entered on a single line with a single space between each (*i.e.*, as a vector). The program then requests the corresponding channels ratios and computes a matrix of channels ratios and counting efficiencies. The user then enters the perfusion rate, in ml/min, the counting time employed for the experimental values to follow and the collecting time for each effluent sample, after which the program requests entry of the radioactive counts obtained from the $HClO_4$ heart extract made at the end of the experiment, the channels ratio of this value and the weight of the heart, and then the dilution factor for this extract. From these values the program computes the total radioactivity, in dpm/g, remaining in the heart at the end of the experiment, converting cpm to dpm by means of a linear interpolation of channels ratio on counting efficiency in the standard matrix. This is achieved by use of a subroutine entitled INTERPOLATE, the listing of which is given at the end of HEFFLUX2. The user then enters the experimental effluent sample counts sequentially, starting at time = 0. Allowance is made for entry on two lines. If only one line is needed, the user types the APL characters ⍳0 followed by "Return" when input on the second line is requested. The program then requests entry of the corresponding channels ratios, the same provision being made for two-line input. If the number of channels ratio values is less than the number of values for the counts, a mean value of channels ratio is computed and the raw counts are converted to dpm using a single value of efficiency determined by interpolation of the channels ratio value in the standard matrix. If the numbers of observations for sample counts and channels ratio are the same, a vector of corresponding efficiencies is computed by interpolation of the sample channels ratios in the standard matrix and the sample dpm values are calculated using the values in this vector. The program then computes the values of efflux in dpm/g/min, by conversion of the raw counts to dpm and making corrections for tissue weight, sample volume, flow rate and collecting period. The total tissue radioactivity at the end of each perfusion period (cumulative dpm/g) is computed by sequentially adding the total efflux to the value for the radioactivity remaining in the heart at the end of the experiment, starting with the last effluent sample (*i.e.*, back addition) and the values for efflux coefficient (% efflux) are computed by dividing each value of efflux by the radioactive content at that time and converting to a percentage. These three data strings, together with the corresponding time are then converted to a matrix and printed out, after which the value of the heart content in dpm/g is printed. The user may then request a graphical printout of the efflux coefficient. If the user wishes to, he may now process other experiments for which he only has to enter the experimental values. In addition to obtaining an individual printout for each experiment, the values for efflux coefficient for each experiment are automatically added and at the end of the last experiment their means are printed along with a graphical representation.

PROGRAM 2. 8K-FORTRAN EFFLUX PROGRAM.

```
DIMENSIONS X(4),Y(4),TPM(200),TPM1(200)
DIMENSIONS TPM2(200),DPM(200)
DO 99 LH=1,10
VRITE(1,10)
10FORMAT(/'INPUT BACKGROUND'/)
READ(1,20) BACKG
20FORMAT(F12.3)
VRITE(1,11)
11FORMAT('INPUT TIME')
READ(1,20) TIME
VRITE(1,12)
12FORMAT('RATE')
READ(1,20) PATE
VRITE(1,13)
13FORMAT('VOLUME')
READ(1,20) VOL
VRITE(1,14)
14FORMAT('COUNTS')
READ(1,20) COUNT
VRITE(1,15)
15FORMAT('VEIGHT')
READ(1,20) VT
VRITE(1,16)
16FORMAT('EFFICIENCY')
READ(1,20) EFF
CRT=(COUNT-BACKG)*500.0/(EFF*VOL)
VRITE(1,17)
17FORMAT('NO. OF POINTS FOP GRAPH')
PEAD(1,21) N
21FORMAT(I2)
VRITE(1,70)
70FORMAT('COOPDINATES')
DO 50 I=1,N
READ(1,22) X(I),Y(I)
22FORMAT(2F8.3)
50CONTINUE
VRITE(1,71)
71FORMAT('NO. OF OBSERVATIONS')
READ(1,21) NUM
VRITE(1,18)
18FORMAT(/'STAPT READEP AND PRESS CONT'/)
PAUSE
READ(1,20) TEMP
KK=0
DO 52 IL=1,NUM
PEAD(1,23)C,P
23FORMAT(15X,F8.1,43X,F5.3)
DO 51 I=1,N
IF(R-X(I))30,30,51
51CONTINUE
30II=I
III=II-1
X0=X(III)
X1=X(II)
Y0=Y(III)
Y1=Y(II)
E=1.0/(X0-X1)*((Y0-Y1)*P-Y0*X1+Y1*X0)
DPM(IL)=(C-BACKG)/E*100.0
TPM(IL)=DPM(IL)*PATE/VOL
DPM(IL)=TPM(IL)/VT
KK=KK+1
IF(KK-NUM)90,52,52
90READ(1,20) TEMP
52CONTINUE
TPM1(NUM)=CRT+TPM(NUM)
TPM2(NUM)=TPM(NUM)/(TPM1(NUM)-(TPM(NUM)/2.0))/TIME
IT=NUM+1
DO 53 I=2,NUM
J=IT-1
TPM1(J)=TPM1(J+1)+TPM(J)
TPM2(J)=TPM(J)/(TPM1(J)-(TPM(J)/2.0))/TIME
53CONTINUE
DO 55 L=1,NUM
TPM(L)=DPM(L)
55CONTINUE
DO 54 I=1,NUM
VRITE(1,60) TPM(I),TPM(I),TPM2(I)
60FORMAT(/E12.5,2X,E12.5,2X,E12.5,2X,E12.5)
54CONTINUE
99CONTINUE
CALL EXIT
END
```

Most of the efflux experiments reported in this study were processed by means of a program written in 8K-FORTRAN for use in a PDP-8 mini-computer. This program (Program 2) performs essentially the same computations as HEFFLUX2, except that input of experimental values is from punched-paper tapes made by the output device (ITT-Teletype) connected to the Beckman LS-230 scintillation counter. Other input, *i.e.*, background counts, collecting time, perfusion rate, sample volume, heart extract counts, heart weight, counting efficiency for the heart extract, the coordinates of the standard quench correction curve and the number of experimental observations is made by the operator at the keyboard. A minor difference between this program and HEFFLUX2 is that the values of efflux coefficients are not given as percentages and are computed on the basis of the radioactivity remaining in the tissue at the middle of each collecting period.

PROGRAM 3. MTS-FORTRAN EFFLUX PROGRAM

```
      DIMENSION X(4),Y(4),TPM(200),TPM1(200),DPM(200),TPM2(200)
      DATA BACKG/12.5/,TIME/2.0/,RATE/4.0/,VOL/0.5/
      DATA N/4/,X/0.554,.641,.680,.785/,Y/50.69,45.54,42.92,35.09/
      WRITE(3,5)
5     FORMAT(/' ENTER NO. OF SETS OF DATA:')
      READ(5,6) NSET
6     FORMAT(I1)
      DO  99 LN=1,NSET
      WRITE(3,10) BACKG
10    FORMAT(/' INPUT BACKGROUND=',E12.4)
      WRITE(3,11) TIME
11    FORMAT(/' INPUT TIME=',E12.4)
      WRITE(3,12) RATE
12    FORMAT(/' RATE=',E12.4)
      WRITE(3,13) VOL
13    FORMAT(/' VOLUME=',E12.4)
      WRITE(3,201)
201   FORMAT(/' ENTER COUNT:')
      READ(5,20) COUNT
20    FORMAT(F12.3)
      WRITE(3,200) COUNT
200   FORMAT(/' COUNT=',E12.4)
      WRITE(3,300)
300   FORMAT(/' ENTER WEIGHT:'/)
      READ(5,20) WT
      WRITE(3,301) WT
301   FORMAT(/' WEIGHT=',E12.4)
      WRITE(3,400)
400   FORMAT(/' ENTER EFFIENCY:')
      READ(5,20) EFF
      WRITE(3,401) EFF
401   FORMAT(/' EFFLUX=',E12.4)
      CRT=(COUNT-BACKG)*500.0/(EFF*VOL)
      WRITE(3,70)
70    FORMAT(/' COORDINATES ARE:'/)
      DO 50 I=1,N
      WRITE(3,500) X(I),Y(I)
500   FORMAT(' ',2F8.3)
50    CONTINUE
      WRITE(3,600)
600   FORMAT(/' ENTER NO. OF OBSERVATIONS:'/)
      READ(5,21) NUM
21    FORMAT(I2)
C     READ DATA FROM PAPER TAPE ON UNIT 1
      KK=0
      DO 52 IL=1,NUM
      READ(1,23) C,R
23    FORMAT(15X,F8.1,43X,F5.3)
      DO 51 I=1,N
      IF(R-X(I)) 30,30,51
51    CONTINUE
30    II=I
      II1=II-1
      X0=X(II1)
      X1=X(II)
      Y0=Y(II1)
      Y1=Y(II)
      E=1.0/(X0-X1)*((Y0-Y1)*R-Y0*X1+Y1*X0)
      DPM(IL)=(C-BACKG)/E*100.0
      TPM(IL)=DPM(IL)*RATE/VOL
      DPM(IL)=TPM(IL)/WT
      KK=KK+1
      IF(KK-NUM) 90,52,52
90     CONTINUE
52    CONTINUE
      TPM1(NUM)=CRT+TPM(NUM)
       TPM2(NUM)=TPM(NUM)/(TPM1(NUM)-(TPM(NUM)/2.0))/TIME
      IT=NUM+1
      DO 53 I=2,NUM
      J=IT-I
      TPM1(J)=TPM1(J+1)+TPM(J)
      TPM2(J)=TPM(J)/(TPM1(J)-(TPM(J)/2.0))/TIME
53    CONTINUE
      DO 55 L=1,NUM
      TPM(L)=DPM(L)
55    CONTINUE
      DO 54 I=1,NUM
      WRITE(6,60) TPM(I),TPM1(I),TPM2(I)
60    FORMAT(/E12.4,2X,E12.5,2X,E12.5,2X,E12.5)
54   CONTINUE
99    CONTINUE
      STOP
      END
```

As a result of a breakdown in the PDP-8 mini-computer, some of the efflux experiments were processed using a program written in MTS-FORTRAN and employing the IBM 360/67 data processing system available in the University of Alberta. This program (Program 3) is almost identical to Program 2. The only essential difference is in the operating procedure involving the reading of punched paper tapes. Each of the data tapes was read in advance and the contents of each allocated to a file, the reference address of which was then specified from the remote terminal by the user in the initial instructions comprising the "Run" command. The operating instructions were also designed to allow the storage of each output set in a temporary file, the contents of which were printed at a remote line-printing station at the end of each session.

TABLE 4. *EFFECT OF SAMPLE VOLUME ON ^3H COUNTING EFFICIENCY*

Volume of Added Solution		Counting Efficiency (Mean % ± S.E.M.; n = 4)		
ml	%	H_2O	Krebs	0.4N $HClO_4$
0	0	49.95 ± 0.70	-	-
0.2	4.3	46.99 ± 0.50	46.96 ± 1.03	44.86 ± 0.94
0.4	8.2	47.14 ± 0.55	46.36 ± 2.68	41.30 ± 1.40
0.6	11.8	46.91 ± 1.59	46.10 ± 0.99	41.37 ± 1.82
0.8	15.1	31.79 ± 1.50	40.08 ± 1.39	38.37 ± 0.68

TABLE 5. EFFECT OF OSMOLARITY AND IONIC STRENGTH ON ^{3}H-NA EFFLUX

	Control	56 mM KCl	90 mM Sucrose	56 mM Choline Chloride
Mean Efflux (pmol/g/30 min)	14.16	31.39	8.74	19.27
S.E.M.	1.73	4.97	1.20	2.54
n	9	16	6	7

TABLE 6. EFFECT OF ANIONS AND CATIONS ON ³H-NA EFFLUX

	KCl	NaCl	KCH_3SO_4	$NaCH_3SO_4$	LiCl
Mean Efflux (pmol/g/30 min)	31.39	31.08	36.27	29.64	24.48
S.E.M.	4.97	4.76	3.53	2.32	3.92
n	16	5	5	5	5

TABLE 7. EFFECT OF K$^+$ CONCENTRATION OF ^{3}H-NA EFFLUX

	TOTAL EXTERNAL CONCENTRATION OF K$^+$ (mM)										
	6	20	34	46	60	76	80	100	116	170	230
^{3}H-NA Efflux (pmol/g/30 min)	14.16	20.89	13.78	29.42	31.39	50.76	47.76	72.85	66.13	70.44	90.26
S.E.M.	1.73	3.44	2.43	4.59	4.97	5.57	9.63	19.34	6.26	14.39	9.54
n	9	6	7	5	16	5	5	4	5	5	5

TABLE 8. *DETERMINATION OF EXTRACELLULAR SPACE WITH ^{14}C-MANNITOL*

TREATMENT	INFUSION TIME (MIN) 1	2	4	8
Normal	37.06 ± 3.64	39.06 ± 0.86	40.34 ± 2.32	40.13 ± 0.81
5 x 10^{-7} M NA	49.83 ± 5.66	89.88 ± 4.99	103.95 ± 17.39	97.36 ± 12.86
112 mM KCl	43.28 ± 1.59	42.69 ± 1.97	48.09 ± 3.00	55.94 ± 3.78

Values are given as mean % ± S.E.M.; n = 5.

TABLE 9. *EFFECT OF TIME ON UPTAKE VELOCITY OF ^{3}H-NA*

	Uptake Time (min)			
	1	2	4	8
Total ^{3}H-NA Uptake (pmol/g)	82.05	23.07	293.4	675.9
S.E.M.	21.06	10.17	36.88	135.3
n	5	5	5	5

TABLE 10. *EFFECT OF K^+ CONCENTRATION ON ^{3}H-NA UPTAKE VELOCITY*

Total $[K^+]_{out}$ (mM)	^{3}H-NA CONCENTRATION (x 10^{-8} M)				
	1.0	1.25	1.67	2.5	5.0
0	3.79±0.50(5)	10.52±0.45(5)	10.88±0.50(5)	20.16±0.59(5)	28.95±0.56(5)
6	6.06±0.57(5)	12.65±1.45(6)	19.55±0.54(5)	28.46±0.45(5)	37.37±2.71(5)
32	4.61±0.68(5)	10.65±0.39(5)	13.76±0.65(5)	15.72±0.98(5)	39.28±1.81(5)
62	4.59±0.49(5)	8.43±0.61(5)	7.32±1.04(5)	17.41±3.11(4)	-
118	3.10±0.53(5)	6.62±0.50(5)	5.19±0.89(5)	11.73±1.90(5)	25.63±1.72(5)
230	3.73±0.86(5)	4.47±0.23(5)	7.68±0.14(5)	7.61±0.35(5)	16.37±1.59(5)

Values are given as mean uptake velocity (pmol/g/min) ± S.E.M. (n).

TABLE 11. *EFFECT OF DMI ON PASSIVE AND KCL EVOKED EFFLUX*

Treatment	Mean Efflux pmol/g/30 min	S.E.M.	n
56 mM KCl	31.39	4.97	16
56 mM KCl + 10^{-7} M DMI	21.40	3.19	6
Control	14.16	1.73	9
10^{-7} M DMI	24.60	5.22	5

TABLE 12. *UPTAKE, RETENTION AND RELEASE OF ^{3}H-NA IN SYMPATHECTOMIZED HEARTS*

	CONTENT		RELEASE	
	t = 0	t = 60	Spontaneous	KCl Evoked
6-OHDA	324.99 ± 67.18(5)	31.63 ± 4.75(5)	2.62 ± 0.417(4)	15.75 ± 1.70(5)
6-OHDA + DMI	332.94 ± 50.47(5)	29.94 ± 5.41(5)	-	17.74 ± 2.18(5)
6-OHDA + SKF-550	385.61 ± 26.56(5)	29.51 ± 3.07(5)	-	17.59 ± 1.09(5)
6-OHDA + DMI + SKF-550	386.77 ± 15.50(5)	21.72 ± 4.06(5)	-	16.53 ± 3.18(5)
Reserpine	214.31 ± 26.06(5)	15.68 ± 3.84(5)	2.56 ± 0.285(8)	14.06 ± 3.15(5)

The values are given as means ± S.E.M. (n). For heart content the values are in pmol/g and for release in pmol/g/30 min.